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Our ref Unser Zeichen	Your ref Ihr Zeichen	Date Datum
OF387	EP 0 656 786	June 9, 2005

European Patent No. 0656786 B1 (Application No.: 93909679.8)
Patentee: Novogen Research Pty. Ltd.
Opponent: Solbar Industries Ltd.
Date of publication and mention of the grant of the patent: September 15, 2004

On behalf of Solbar Industries Ltd., Hahadarim Street 2, Heavy Ind. Estate, Ashdod, 77613, Israel, we herewith file

Opposition

against above identified European Patent (the Patent) and request complete revocation of the Patent based on Art. 100(a) EPC because of lack of novelty (Art. 52(1) together with Art. 54 EPC) and/or lack of inventive step (Art. 22(1) together with Art. 56 EPC), and/or based on Art. 100(c).

The opposition fee in the amount of EUR 610,00 is paid by the enclosed check. The power of attorney is also enclosed herewith.

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In case that the Opposition Division is not intending to completely revoke the Patent on the basis of the documents of prior art and the arguments brought forward in this Opposition in the written procedure, we herewith request oral proceedings according to Art. 116 EPC.

Grounds of Opposition

I.

The following documents of prior art will be used in this Opposition:

D1: Adlercreutz et al., "Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet", Am. J. Clin Nutr., Vol. 54, pp. 1093-1100 (1991).

D2: Cassady et al., "Use of a Mammalian Cell Culture Benzo(α)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from Trifolium pratense L", Cancer Research, Vol. 48(22), pp. 6257-6261 (1988).

D3: JP 61-246124 A (Abstract and English language translation)

D4: JP 01-258669 A (Abstract and English language translation)

D5: Rami S. Kaldas et al., "Reproductive and General Metabolic Effects of Phytoestrogens in Mammals", Reproductive Toxicology Review, Vol. 3, No. 2, pp. 81-89 (1989).

D6: Mark Messina et al., "The Role of Soy Products in Reducing Risk of Cancer", J. of National Cancer Institute, Vol. 83, No. 8, pp. 541-546 (1991).

D7: Mark Messina et al., „Increasing use of soyfoods and their potential role in cancer prevention", Perspectives in Practice, Vol. 91, p. 836-840 (1991).

D8: Gisela Wilcox et al., "Oestrogenic Effects of Plant Foods in Postmenopausal Women", British Med. J., 301, pp. 905-906 (1990).

D9: Nancy Beckham, "Menopause", The Family Guide to Natural Therapies, Greenhouse Publications, pp. 41-42, 50 (1988).

D10: Nancy Beckham, "Herbal Help to Avoid Menopause Symptoms", Australian Wellbeing, No. 29, pp.74-76 (1988).

D11: Statutory Declaration of Ms. Nancy Beckham.

D12: Arthur C. Eldridge, "Determination of Isoflavones in Soybean Flours, Protein Concentrates, and Isolates", J. Agric. Food Chem. Vol. 30, pp. 353-355, (1982).

II.

According to its patent claim 1 (only independent claim), the Patent is directed to

the use of an isoflavone phyto-oestrogen extract of soy or clover for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with menopause or prostate cancer.

III.

All features of the main claim of the Patent (claim 1) are known from the prior art.

Document **D1** discusses urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming traditional Japanese diet. In the abstract, it is mentioned that the excretion of isoflavonoids correlated with soybean-product intake, and that the low mortality in breast and prostate cancer in Japanese women and men, respectively, may be due to the high intake of soybean products. A more detailed discussion of the role of the phytoestrogens can be found in the passage starting in the middle of the right column of p. 1097 to the end of the article. It is especially discussed that soybean products could be protective against prostate cancer and this is related to the very large amounts of isoflavonoids in such products, mainly genistein, daidzein and equol.

Therefore, someone skilled in the art, i.e. an expert in phytopharmaceuticals, would have been prompted by the disclosure of document **D1** to use soybean products, and in particular an isoflavone phytoestrogen extract of soy for the manufacture of a medicament for the treatment of prostate cancer (one of the medical indications of claim 1 of the Patent).

Document **D2** discusses the potential anticarcinogetic effect of biochanin A, in isoflavone from Trifolium pratense (i.e. red clover). Various in vitro tests were performed to show such effect, and

a respective extract from red clover, i.e. in isoflavone phyto-estrogen extract of clover in the meaning of claim 1 of the Patent, has been used and found to be active (p. 6258).

Therefore, someone skilled in the art would have been prompted by the disclosure of document **D2** to try an extract of red clover for the manufacture of a medicament for the treatment of prostate cancer.

Document **D3** discloses a carcinostatic agent whose active principle is 5,7,4′-trihydroxyisoflavone (genistein). Genistein is mentioned in the specification of the Patent as one of the isoflavones which have been identified in respective extracts of soy or clover. This is confirmed in document **D3** under the heading "Prior Art".

Document **D3** describes the carcinostatic properties (and toxicity) of genistein in detail and provides for results of experiments on the inhibition of the propagation and inhibition of the synthesis of DNA in a number of experimental tumor cells. Moreover, the inhibiting action against tyrosine-specific phosphorylase which is believed to contribute to the propagation of carcinoma cells, is shown in document **D3**. On page 11 of document **D3** it is described to administer genistein in unit dosage form.

Although document **D3** does not explicitly describe the use of an isoflavone phyto-oestrogen extract of soy or clover explicitly, it describes the use of one of the major active components thereof. Attention of the Opposition Division is drawn to claim 7 of the patent where genistein is explicitly mentioned.

Therefore, someone skilled in the art would have been prompted by the disclosure of document **D3** to try the claimed extract (instead of one of its single components) for the manufacture of a medicament for administration in unit dosage form for the treatment of prostate cancer.

In the examination procedure the Patentee has argued that disclosure of document **D3** would not have led to the teaching of claim 1 of the Patent, basically with the argument that prostate cancer is very specific and that carcinostatic activity would not have been indicated for the use for this specific type of cancer. However, the whole argumentation was based on the very restricted disclosure in the abstract of document **D3** only. It did not take into account the much more detailed disclosure in the complete document which gives evidence for various carcinostatic activities of genistein against a number of different carcinoma cells and also inhibition activity against a number of enzymes. In view of this much more detailed disclosure, we believe claim 1 to lack inventive step over the disclosure of document **D3**.

Document **D4** discloses a method of manufacturing isoflavone compounds by extraction from soybeans or soybean meal. On page 2 of document **D4** the isoflavone compounds of such soybean extracts are described as having, amongst others, cancer-controlling effect as inducing the differentiation of cancer cells and preventing the cancer genes.

Although **D4** does not explicitly describe the use of soy extract for the manufacture of a medicament for administration in unit dosage form for the treatment of prostate cancer, the disclosure of document **D4** would have led someone skilled in the art to try such extract for this medical indication.

Again, only the abstract of this document (as D26) has been discussed in the examination procedure, but not the complete disclosure of this document which is now herewith submitted in English language translation.

Document **D5** describes the reproductive and general metabolic effects of phyto-oestrogens in mammals. On page 82, column 1 and column 2, in particular Table 1, it identifies common plants that contain such substances. These plants include clovers, soybeans and soya sprouts. Therefore, it is obvious that such substances are contained in an isoflavone phyto-oestrogen extract of soy or clover. At page 88, column 2, it is disclosed that phyto-oestrogens could be used to reduce cancer of hormone-responsive tissues (lines 13 to 14). Although not explicitly mentioned, prostate cancer is exactly that type of cancer for which activity is described in document **D5**. At the same page (lines 31 to 37), it is disclosed that phyto-oestrogens could also alleviate vasomotor symptoms in menopausal women.

Therefore, someone skilled in the art would have deducted from document **D5** that an extract of soy or clover could be used as a medicament for the treatment of symptoms associated with menopause and/or prostate cancer.

Document **D6** discusses the role of soy products, and in particular the specific role of isoflavones therein, in cancer prevention (p. 541 and 542). The article also discusses studies of the effects of feeding soy to postmenopausal women (p. 542, bottom of left column). On pages 544 and 545 of the article the soybean processing, and in particular solvent extraction is described as a primary method of producing soybean products for such uses.

Document **D7** is also directed to the potential role of soyfoods in cancer prevention. Starting from p. 837, bottom of right column, experimental studies on the effect of isoflavones contained in soy-

bean product are discussed. On page 838, bottom of left column, it is mentioned that "the effects of isoflavones may not be limited solely to hormone-related cancers", and on the same page, top of right column, it is stated: "Consequently, isoflavones may have a role to play in the prevention of a wide range of cancers". A little bit later on the same page, several studies are mentioned looking specifically at the estrogenic/antiestrogenic effects of soybeans in postmenopausal women.

Document **D8** relates to the estrogenic effects of plant foods in postmenopausal women. The experiments included the use of soy flour and red clover sprouts as a food supplement taken over a six week period.

Document **D9**, which are copies from a book related to natural therapies, discusses the use of a number of estrogenic plants for the treatment of symptoms associated with menopause. Amongst others, red clover and soybeans are mentioned.

In another publication of the same author (document **D10**), the contents of estrogens in foods and herbs, and amongst others in red clover and soybeans, are discussed in the context of how to help to avoid menopausal symptoms.

Documents **D8 – D10** at least taken together and/or in combination with any of documents **D1** to **D7** can also be seen as evidence of lack of inventive step of subject-matter of claim 1 of the Patent.

IV.

In addition to the documents of prior art discussed under III., there is another valuable source of information which clearly shows lack of novelty and/or inventive step of subject-matter of claim 1 of The Patent, namely the use of soy and clover extracts for treating patients, in particular with

menopausal symptoms, but also for other conditions relating to imbalances of female hormones, treating prostate problems and as an adjunct therapy for cancer, by natural therapists. We herewith submit a copy of the Statutory Declaration of Ms. Nancy Beckham (**D11**), a practicing natural therapists since the early 1980's which has been filed in the Opposition Procedure against the corresponding Australian Patent. In this Statutory Declaration, which is very concise and self-explanatory, Ms. Beckham in detail describes her interest in isoflavonic phyto-oestrogens known from clover and her use of such phyto-oestrogens in respective treatments. She also published an article and a book (both from 1988) describing the use of oestrogen containing plants including red clover and soybeans for the treatment of menopausal symptoms which are discussed herein above as **D9** and **D10**. In Ms. Beckham's Statutory Declaration also a number of documents are mentioned which directly or indirectly reflects the role of phyto-oestrogens for the human health.

The Statutory Declaration of Ms. Nancy Beckham therefore gives evidence that isoflavonic phyto-oestrogens as obtained in extracts of clover and soy, have been used, at least in Australia, by natural therapists, at least for the treatment of menopausal symptoms. Therefore, the subject-matter of claim 1 of the Patent lacks novelty and/or inventive step over the described public prior use in Australia.

V.

Claim 2 does not add anything which could form a basis for a patentable claim, as the formulation of a medicament with at least one dietary suitable excipient is a trivial measure in pharmaceutical technology.

Claims 3 and 5 just specify the two different alternatives of claim 1, namely to use either soya or clover as the source for the isoflavone phyto-oestrogen extracts and therefore add nothing substantial.

Claim 4 specifies that the isoflavone phyto-oestrogen is extracted from soya hypocotyls. This is one obvious alternative for someone skilled in the art for the preparation of a soy extract and does therefore add nothing substantial.

Claim 6 does not specify any additional features as it is just a description of what phyto-oestrogens may be present in the respective extracts. This has already been described in the specification itself as known from prior art. One example of prior art which explicitly shows the phyto-oestrogens contained in soybean extract is document **D11**.

Claim 7 does not add as being substantial to claim 6.

Claim 8 specifies the amount of isoflavone phyto-oestrogens to be administered. The ranges given at least overlap with the ranges given for the administration of genistein in document **D3** (page 10).

Also the at least daily administration of the medicament as claimed in claim 9 can be found at page 10 of document **D3**.

It is difficult to see that claims 10 or 11 would add anything substantial which could form the basis for an allowable claim.

VI.

Claim 1 of the Patent clearly goes beyond the original disclosure of the Patent. In the publication of the Application (WO93/23069), the products of the Application which are described to be either an extract from red clover (Example 1) or soya hypocotyls (Example 3) are not disclosed for the specific medical indications which are claimed in claim 1 of the Patent. In the bridging paragraph between pages 15 and 16, it is stated, under (ii) that the product of the application would reduce the risk of development of cancer of the prostate. This, however, would be the medical indication of the prevention of prostate cancer, but not of the treatment thereof, as presently claimed. Under (vi), the product of the application is described to reduce the risk of development of pre-menstrual syndrome, and, under (vii), symptoms associated with menopause. Again, there is only a disclosure of the prevention of such symptoms, but not of the treatment thereof as presently claimed.

We therefore believe claim 1 to go beyond the original disclosure of the application. We would like to draw the attention of the Opposition Division to the fact that, during the last phase of the examination procedure, prevention of the respective diseases or conditions has been deleted from claim 1, as the Patentee was only able to provide for evidence of the effectiveness of the extracts in treatments. As this use is, however, not based on the original disclosure, granted claim 1 of the Patent is not valid.

VII.

Summarizing the above arguments, it has become clear that the Patent is to be revoked both based on Art. 100(a) EPC because of lack of novelty (public prior use in Australia (**D11**)) or lack of inventive step (in view of documents **D1** to **D10**) and/or based on Art. 100(c) because of lack of dis-

closure of the claimed medical indication of treatment of pre-menstrual syndrome, symptoms associated with menopause or prostate cancer.

FORRESTER & BOEHMERT

Dr. Andreas Winkler
Patent Attorney

Enclosure:
5 Copies of this letter
Check in the amount of EUR 610,00
Power of Attorney
D1: Adlercreutz et al., "Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet", Am. J. Clin Nutr., Vol. 54, pp. 1093-1100 (1991), 6-fold
D2: Cassady et al., "Use of a Mammalian Cell Culture Benzo(α)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from Trifolium pratense L", Cancer Research, Vol. 48(22), pp. 6257-6261 (1988), 6-fold
D3: JP 61-246124 A (Abstract and English language translation), 6-fold
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D5: Rami S. Kaldas et al., "Reproductive and General Metabolic Effects of Phytoestrogens in Mammals", Reproductive Toxicology Review, Vol. 3, No. 2, pp. 81-89 (1989), 6-fold
D6: Mark Messina et al., "The Role of Soy Products in Reducing Risk of Cancer", J. of National Cancer Institute, Vol. 83, No. 8, pp. 541-546 (1991), 6-fold
D7: Mark Messina et al., „Increasing use of soyfoods and their potential role in cancer prevention", Perspectives in Practice, Vol. 91, p. 836-840 (1991), 6-fold
D8: Gisela Wilcox et al., "Oestrogenic Effects of Plant Foods in Postmenopausal Women", British Med. J., 301, pp. 905-906 (1990), 6-fold
D9: Nancy Beckham, "Menopause", The Family Guide to Natural Therapies, Greenhouse Publications, pp. 41-42, 50 (1988), 6-fold
D10: Nancy Beckham, "Herbal Help to Avoid Menopause Symptoms", Australian Wellbeing, No. 29, pp.74-76 (1988), 6-fold
D11: Statutory Declaration of Ms. Nancy Beckham, 6-fold
D12: Arthur C. Eldridge, "Determination of Isoflavones in Soybean Flours, Protein Concentrates, and Isolates", J. Agric. Food Chem. Vol. 30, pp. 353-355, (1982), 6-fold

EINZELVOLLMACHT / SPECIFIC AUTHORISATION

Zeichen des Vertreters (der Vertreter) (max. 15 Positionen) Representative's Reference (max. 15 spaces)	Nr. der Anmeldung (des Patents) Application/Patent No. EP 0 656 786 B1

Ich (Wir) / I (We)

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Datum / Date 6.06.05

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Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet[1-4]

Herman Adlercreutz, Hideo Honjo, Akane Higashi, Theodore Fotsis, Esa Hämäläinen, Takeshi Hasegawa, and Hiroji Okada

ABSTRACT Epidemiologic studies revealed low mortality in hormone-dependent cancer in Japanese women and men consuming a traditional diet. We previously found that certain diphenolic food components, lignans and isoflavonoids, which are converted to biologically active hormone-like substances by intestinal microflora, may be cancer-protective agents. Therefore, we studied urinary excretion of these compounds (enterolactone, enterodiol, daidzein, equol, and *O*-desmethylangolensin) in 10 women and 9 men in a rural village south of Kyoto, Japan. The subjects consumed a typical low-fat diet with much rice and soy products, fish, and vegetables. An isotope-dilution gas chromatographic–mass spectrometric method was used for the assays. The urinary excretion of lignans was low but that of the isoflavonoids was very high. The excretion of isoflavonoids correlated with soybean-product intake. The low mortality in breast and prostate cancer of Japanese women and men, respectively, may be due to the high intake of soybean products. *Am J Clin Nutr* 1991;54:1093–1100.

KEY WORDS Japanese, diet, urine, lignans, isoflavonoids, enterolactone, enterodiol, daidzein, equol, genistein, *O*-desmethylangolensin, soybean, gas chromatography, mass spectrometry, sex-hormone–binding globulin

Introduction

Mammalian lignans and isoflavonoid phytoestrogens, occurring in all studied animal and human biological fluids and in feces, are diphenolic compounds with molecular weights similar to those of steroid estrogens (1–3). Precursors in plants seem to occur as glycosides (4, 5), and the mammalian compounds are produced from plant lignans and isoflavonoids by intestinal microflora (6–8). Most of the original plant aglycones, such as formononetin, matairesinol, and secoisolariciresinol, occur only in very low concentrations in urine (9, 10). All compounds investigated so far are weakly estrogenic but have shown many other biological activities, producing antiestrogenic (1–3); antiviral (11, 12); and antiproliferative, cytotoxic, and growth-inhibiting effects (3, 13–15). Studies indicate that they most likely stimulate the production of sex-hormone–binding globulin (SHBG) in the liver (2, 14–18) and may in this way significantly influence biological activity of the sex hormones. The higher SHBG values seen in vegetarians (2, 17–19) are probably due to the effect of these diphenolic compounds on liver synthesis of the protein (14). Studies in both young and old women with breast cancer and in various dietary groups indicate that urinary excretion of these compounds is highest in vegetarians and lower in omnivores and breast-cancer patients (2, 18, 20). It was shown that their urinary excretion correlates with the intake of fiber-rich food (2, 17, 18).

Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the original traditional Japanese diet have low breast-cancer incidence and mortality (21–24). Similarly, Japanese men have low mortality with prostate cancer, although autopsy studies have found that the incidence of prostate cancer in Japanese and Western men are similar (25–27). These cancers are sex-hormone dependent and could potentially be influenced both by alterations of sex-hormone metabolism caused by lignans and isoflavonoids or by a direct effect of these compounds on their growth. Because of the associations between diet and these diseases, we decided to study the urinary excretion of lignans and isoflavonoid phytoestrogens in groups of Japanese men and women consuming a traditional diet. A preliminary report was published as an abstract (28).

Subjects and methods

Participants

The subjects participating in this investigation were apparently healthy and were recruited in a small rural village south of Kyoto,

[1] From the Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, Helsinki, Finland, and the Departments of Obstetrics and Gynecology and Preventive Medicine and the Laboratory of Gas Chromatography-Mass Spectrometry, Kyoto Prefectural University of Medicine, Kyoto, Japan.

[2] Preliminary report published as an abstract.

[3] Supported by Sigrid Jusélius and Finnish Cancer Foundations and the Medical Research Council of the Academy of Finland.

[4] Address reprint requests to H Adlercreutz, Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, SF-00290 Helsinki, Finland.

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Japan. Two of the women were found to have hypertension (blood pressure 146/96 and 180/100, respectively). Most of the participants were farmers cultivating tea and rice. Originally 10 men and 10 women volunteered for the study, but 1 man was dropped because his urine volume was not known. Their main work was in agriculture and they consumed mainly their own products. The ages of the men and women were 50.4 ± 18.0 and 46.8 ± 11.5 y, respectively. Height, weight, and body mass index [BMI, in weight (kg)/height $(m)^2$] were, respectively, 160.8 ± 7.8 cm, 58.6 ± 5.8 kg, and 22.7 ± 2.3 for men and 153.1 ± 6.5 cm, 52.9 ± 7.2 kg, and 22.6 ± 3.5 for women. All subjects were within 15% of normal weight.

Collection of samples

Urine was collected for 48 h in plastic bottles containing 2 g ascorbic acid. The bottle was kept in a cool place during collection. The urine was mixed and measured and a sample was frozen as soon as possible and transported to Finland in dry ice for analysis.

Dietary data

The study was carried out in October 1985. Before the survey a nutritionist explained how to weigh the food components and how to write down the results on a form. Most of the food was weighed. Some food, such as bread and milk, was recorded as a piece of bread or cup of milk and the nutritionist estimated the weight of these food items afterwards. Food intake was recorded for 3 d and the nutritionist followed all subjects every day during the survey period. Calculation of the food data was made by an experienced nutritionist using the *Standard Tables of Food Composition in Japan* (29); for fiber calculations the *Food Composition Tables of Dietary Fibers, Minerals, Cholesterol, Fatty Acids* was used (30). The amount of soy sauce in the diet was calculated from the total sodium chloride content of the urine. According to earlier studies Japanese obtain 25.8% of their sodium chloride from soy sauce (31). Soy sauce contains 15% NaCl. The consumption of soy sauce is estimated by using the following formula:

$$\text{Soy sauce} = (\text{amount of NaCl in urine}) \times 0.258/0.15$$

This is the traditional way to estimate soy sauce consumption in Japanese subjects because they do not add any other salt to their food. It is an estimate and not an exact figure and the values were not included in the correlation analyses.

Analytical method

The trivial and systematic names of the compounds measured and discussed are as follows [structures were shown previously (3)]: enterolactone (Enl), *trans*-2,3-bis[(3-hydroxyphenyl)methyl]-γ-butyrolactone; enterodiol (End), 2,3-*bis*[(3-hydroxyphenyl)-methyl]-butane-1,4-diol; daidzein (Da), 4',7-dihydroxyisoflavone; equol (Eq), 4',7-dihydroxyisoflavan; *O*-desmethylangolensin (*O*-Dma), 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-propan-1-one.

The method used was a modification of a method for determining the estrogen profile in urine by ion-exchange chromatography and capillary gas chromatography–mass spectrometry in the selected ion-monitoring mode (GC-MS-SIM, or GC/MS) (32–34). Originally, estrogens also were determined but because of very low concentrations of some fractions, the amount of urine saved for the purpose was too small and the analyses could not be repeated. Therefore, only the lignan and isoflavonoid values are presented. Only modifications of the method are described.

Protection of the carbonyl functions by ethoximation (necessary only for the estrogens), and extraction with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) were carried out as described (33, 34). The removal of inhibitors of the enzyme hydrolysis by ion-exchange chromatography on a DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column in the acetate form was done in a smaller column (0.5 × 3 cm instead of 0.5 × 5 cm). For hydrolysis and purification of the hydrolysate, before evaporation of the last fraction obtained from the above DEAE-Sephadex column, the following deuterated internal standards were added to the eluate: d_6-Enl and -End, d_4-Da and -Eq, and d_3-*O*-Dma (35, 36). This was followed by hydrolysis and Sep-Pak extraction; application of the methanolic extract directly on the QAE-Sephadex A-25 in the acetate form (0.5 × 5 cm); and elution of the estrogens, lignans, and Eq with 4 mL methanol as described. The modification in this step is that *O*-Dma and Da are eluted after this with 4 mL 0.2 mol acetic acid/L in methanol. This fraction is then, after evaporation of the solvent, ready for derivatization (trimethylsilyl ethers) and GC/MS. Selective fractionation of estrogens with vicinal *cis*-hydroxyls was carried out in a borate column with new dimension (0.5 × 3 cm instead of 0.5 × 2.5 cm). Elution of the diphenols was carried out as described and this fraction contains the isoflavan Eq and the two lignans Ent and End.

The two fractions containing lignans and isoflavonoid phytoestrogens and their deuterated internal standards are converted to their trimethylsilyl ether (TMS) derivatives (32) and quantified by GC/MS by using the following ion pairs (mass/charge): Eq, 386/390; Da, 398/402 (and 383/387); End, 410/416; Enl, 442/448; and *O*-Dma, 459/464 (36). The measurements were carried out with a Hewlett-Packard 5995 B GC/MS (Avondale, PA) instrument equipped with a Pascal work station and with an automatic injector.

Urinary excretion of < 0.0025 μmol/d cannot be measured, and between 0.0025 and 0.005 μmol/d the method must be regarded as semiquantitative. The mean values and interassay imprecision for the control pooled-urine sample, measured 59 times in single assays during 1 y, were as follows: Enl, 3.65 μmol/d (CV 7.4%); End, 0.364 μmol/d (CV 11.6%); and Eq, 0.042 μmol/d (CV 9.4%). For Da at a concentration of 0.028 μmol/d, the interassay imprecision is 11.0% (n = 14) and for *O*-Dma at the high concentrations in this study, the interassay imprecision is 8–10% (CV).

The samples were analyzed in two batches and the values for the control sample were almost identical both times and the same as in analyses before and after these two batches.

Statistical methods

The food data are presented as arithmetic means (±SD) and the lignan and phytoestrogen results as arithmetic means (±SD) and geometric means. Geometric means were used when necessary because of skewness of the distribution of the results. The statistical analyses were carried out by using the *StatView* program for Macintosh (Abacus Concepts, Berkeley, CA). The degree of univariate associations between two variables were estimated as Pearson's correlation coefficients (r). The pairs of

TABLE 1
Intake of various food stuffs by the Japanese women and men consuming a traditional Japanese diet*

Nutrient	Women (n = 10)	Men (n = 9)
	g/d	
Rice	578.5 ± 222.5	764.7 ± 240.3
Wheat	59.5 ± 46.0	139.0 ± 113.6
Potato	62.6 ± 30.2	55.2 ± 34.6
Sugar	8.1 ± 7.0	8.1 ± 7.4
Fats	13.1 ± 7.6	12.7 ± 6.9
Pulses and beans	56.5 ± 36.0	40.9 ± 32.0
Fruit	228.2 ± 111.9	146.9 ± 114.0
Green and yellow vegetables	60.6 ± 33.3	55.7 ± 35.2
Other vegetables	139.3 ± 69.3	130.9 ± 77.2
Pickles	32.9 ± 24.9	23.2 ± 21.2
Algae	1.8 ± 2.0	0.7 ± 0.7
Fish	98.7 ± 46.6	113.6 ± 56.5
Meat	37.0 ± 30.1	73.6 ± 58.4
Eggs	38.4 ± 16.6	57.4 ± 30.6
Milk	112.7 ± 131.0	90.9 ± 90.2
Beer	5.1 ± 16.1	454.6 ± 647.1

* $\bar{x}$ ± SD.

adjusted group means for the two groups studied (women and men) were compared by nonpaired t test.

Results

The intake of various types of food are shown in Table 1, and Table 2 shows the results of the calculations with regard to energy;

TABLE 2
Energy intake, intake of various nutrients, and some ratios in the two study groups*

Nutrient	Women (n = 10)	Men (n = 9)
Energy		
(MJ/d)	8.29 ± 1.64	10.79 ± 3.48
(kcal/d)	1973 ± 391	2569 ± 829
Animal protein (g/d)	35.3 ± 13.9	47.8 ± 18.9
Vegetable protein (g/d)	38.2 ± 10.1	45.1 ± 10.6
Total protein (g/d)	73.6 ± 12.2	93.0 ± 28.4
Carbohydrates (g/d)	311.4 ± 77.0	383.3 ± 100.6
Total fat (g/d)	44.4 ± 14.4	51.0 ± 25.9
Total fiber (g/d)	16.9 ± 4.9	15.3 ± 6.0
Animal protein (%)†	47.2 ± 15.9	49.8 ± 7.9
Proteins (%)‡	15.2 ± 2.1	14.6 ± 1.5
Carbohydrates (%)‡	64.6 ± 6.8	68.2 ± 5.1
Fats (%)‡	20.3 ± 5.5	17.2 ± 4.9
Fat (g/kg body wt)	0.86 ± 0.31	0.85 ± 0.37
Fiber		
(mg/J)	2.1 ± 0.7	1.5 ± 0.7
(g/1000 kcal)	8.8 ± 3.0	6.4 ± 3.0
Fiber (g/kg body wt)	0.33 ± 0.10	0.26 ± 0.09
Fat-fiber ratio	2.5 ± 0.9	2.4 ± 0.9

* $\bar{x}$ ± SD.
† Percent of total protein.
‡ Percent of energy.

TABLE 3
Dietary intake of soy products by the two groups studied*

Soy product	Women (n = 10)	Men (n = 9)
	g/d	
Tofu (soybean curd)	25.0 ± 22.9	18.7 ± 28.8
Miso (bean paste)	12.5 ± 6.2	8.5 ± 6.4
Aburaage (fried thin tofu)	2.6 ± 3.6	3.7 ± 4.2
Atuage (fried thick tofu)	4.0 ± 12.7	0.8 ± 2.3
Koridofu (dried soybean curd)	0.37 ± 0.78	0.07 ± 0.2
Fermented soybeans	2.4 ± 4.5	0.9 ± 2.8
Boiled beans	7.7 ± 17.8	6.5 ± 7.8
Soy sauce	22.9 ± 6.1	19.2 ± 4.7
Soy products (sauce excluded)	54.4 ± 34.3	39.2 ± 36.4

* $\bar{x}$ ± SD.

animal and vegetable protein; total proteins, carbohydrates, fats, and fiber; percentage animal protein and percentage protein; and carbohydrate and fat as percent of total calories. Furthermore, we calculated the fat intake per kilogram body weight, fiber intake per J (per 1000 kcal), and the fat-fiber ratio (Table 2). The diet was a low-fat (fat 17.2% and 20.3% of total calories for men and women, respectively), low-animal-protein diet with moderate amounts of fiber and a low fat-fiber ratio, which is typical for the traditional Japanese diet (37).

Table 3 shows the dietary intake of soy products, which were expected to be the most important source of precursors for the urinary isoflavonoids (3).

Table 4 shows the mean excretion values for the two lignans and three isoflavonoid phytoestrogens. The results show a relatively low excretion of enterolactone, a normal excretion for enterodiol, and a very high excretion of isoflavonoid phytoestrogens. The individual results showed large variation, particularly for equol (from 0 to 10.95 μmol/d). For comparison note that the geometric mean values in young omnivorous women living in Helsinki and in Boston for enterolactone, enterodiol, daidzein, equol, and O-desmethyl-angolensin were 2.46, 0.20, 0.22, 0.10, 0.03, and 2.05, 0.28, 0.32, 0.07, and 0.03 μmol/d, respectively (2).

TABLE 4
Urinary excretion of lignans and isoflavonoid-phytoestrogens in Japanese women and men consuming traditional Japanese diet*

Compound	Women (n = 10)	Men (n = 9)
	μmol/d	
Enterolactone	1.4 ± 1.4 (0.89)	1.1 ± 0.7 (0.89)
Enterodiol	0.7 ± 1.3 (0.41)	0.4 ± 0.3 (0.22)
Total lignans	2.1 ± 2.6 (1.38)	1.5 ± 0.9 (1.13)
Daidzein	2.6 ± 4.0 (2.55)	2.2 ± 2.0 (1.45)
Equol	2.6 ± 4.0 (0.56)	3.0 ± 4.6 (0.54)
O-desmethylangolensin	0.7 ± 0.6 (0.51)	0.2 ± 0.3 (0.11)
Total isoflavonoids	6.9 ± 6.8 (4.73)	3.9 ± 3.3 (2.57)
Total diphenols	9.1 ± 9.3 (6.7)	5.4 ± 4.0 (4.1)

* $\bar{x}$ ± SD (geometric $\bar{x}$).

Table 5 presents a correlation matrix of various food components and urinary excretion of lignans and isoflavonoids in the total material of 19 subjects for whom both food and phytoestrogen data were available.

Discussion

In a previous study of oriental immigrant women from southeast Asia residing in Hawaii (38), the diet was similar to that consumed by the men and women in the rural village in Japan. In the present study the women had a greater energy intake (an additional ~2.1 MJ/d, or 500 kcal/d), which may be due to a physically more active life. However, the percentage intake of calories as fat and the dietary fiber and fat-fiber ratio were very similar to the corresponding values in the previous study. Except for the energy intake the values are very different from those seen in Western societies where the fiber intake is similar but the fat-fiber ratio is much higher. Women living in the Boston area had a fat-fiber ratio of 7.7 for the premenopausal women and 4.6 for the postmenopausal women compared with 2.5 for the women in the present study (39).

With regard to protein intake, expressed as g/d and as percentage of calories, the mean values in the present study were similar and slightly lower, respectively, than those of the immigrants from southwest Asia (38).

Our results are in good agreement with those from an earlier study of 300 female agricultural workers from 18 regions in Japan (37) except for dietary fiber intake, which was much lower (between 5 and 6 g/d) in the women in the earlier study (which may represent crude fiber intake). However, according to the national nutrition survey in Japan, the dietary fiber intake was 22.8 g/d in 1951 and decreased year by year to 17.4 g/d in 1985. These figures are in better agreement with our results obtained in 1985, which show a mean dietary fiber intake in the whole group of ~16 g/d. This latter value is also in good agreement with the value of 13 g/d for nonstarch polysaccharides found by analyses of the Japanese diet in another study (40). On the basis of these investigations and the present investigation, it may be concluded that the amount of dietary fiber in a traditional oriental diet is comparable with that in many Western societies (38–40). We may also conclude that the diet of our subjects was typical for a rural area, where the people to a large extent consume their own products and have a traditional Japanese diet.

The urinary excretion of Enl was, with few exceptions, low in both men and women (Tables 4 and 1A) and was the same as found for the postmenopausal breast-cancer patients in Boston (20). We found a weak correlation between intake of green and yellow vegetables and excretion of Enl and total lignans (Table 5) but no correlation with rice intake. Because these subjects consumed large amounts of rice, it seems justified to conclude that refined rice contains very low amounts, if any, of lignan precursors. There was a better correlation with the intake of soybeans, which thus also may be a source of Enl precursors (Table 5). It is known that soy sauce contains coniferyl alcohol the building block for lignans and lignin (41). The excretion of the lignan End was also found to be associated with the intake of beans and pulses and soy products in general (Table 5).

The excretion of the isoflavonoid phytoestrogens is very high in these Japanese men and women compared with values obtained in women living in Boston (2, 20) and in the Helsinki area (2, 18). The Japanese women in the present study excrete 10 times more Da and 20–30 times more Eq and *O*-Dma than did omnivorous and lactovegetarian women living in the abovementioned two cities. Of the 19 subjects, 47% and 89% excrete micromole amounts of Eq and Da per day, respectively, a phenomenon very rarely seen in subjects consuming a Western diet but seen in subjects consuming a macrobiotic diet (2). The values in an additional study group of nine subjects, including three children (*see* Appendix A), were not significantly different from those in the two main groups (Tables 4 and 1A); they were in fact surprisingly identical. The excretion of matairesinol, the precursor lignan for enterodiol, was very low, but genistein excretion was very high. Genistein is the center of interest in many laboratories because of its very interesting antiproliferative and

TABLE 5
Correlation matrix of various food components and urinary excretion of ligans and isoflavonoids in the whole material (n = 19)

Nutrient	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Total isoflavonoids	Total diphenols
Green and yellow vegetables	0.525*		0.460*					
Pulses and beans		0.541*	0.492*	0.679†	0.737†	0.617†	0.668†	0.693†
Algae				0.561*			0.450‡	0.430‡
Total fat					0.584†			
Percent fat calories					0.469*			
Fat-fiber ratio					0.507*			
Meat					0.507*			
Soy products (not sauce)		0.481*		0.583†	0.746§	0.601†	0.585†	0.588†
Boiled soybeans	0.758§	0.892§	0.849§	0.632†	0.693§		0.757§	0.801§

* $P < 0.05$.
† $P < 0.01$.
‡ $0.05 < P < 0.10$.
§ $P < 0.001$.

antimitogenic effects (*see* below); genistein showed the highest concentration of all phytoestrogens in urine in these nine subjects. The mean value was almost 6 μmol/d and a value as high as 15.5 μmol/d was observed. Also in this smaller group most variation in the excretion values was found for Eq (from 0.01 to 9.16 μmol/d). In 21.4% of all subjects, equol excretion did not significantly differ from zero: this group included two of the three children; the mother of these two children did not excrete equol in significant amounts.

The low excretion of Enl in the Japanese subjects compared, eg, with Finnish women (2), is most likely due to low intake of grain (whole-grain) products such as bread (2, 17, 18, 42, 43). The precursors of the mammalian lignans seem to be located in the aleuronic layer of the grain close to the fiber (15) but definite evidence for this location has not yet been obtained. The mean End values are similar to those observed in lactovegetarian American and Finnish women and higher than in the omnivorous women from the same countries (2, 20). It is likely that the majority of the lignans in these Japanese subjects is derived from nongrain plant products (pulses and beans), as suggested by the correlations found in Table 5.

Eq excretion correlated positively with the intake of total fat ($P < 0.01$), fat-fiber ratio ($P < 0.05$), and meat ($P < 0.05$) and deviated in this aspect from all the other isoflavonoids. Some subjects are not able to produce Eq at all, as also shown previously for nonJapanese subjects (44). It is possible that those consuming more fat and meat have an intestinal flora more capable of producing Eq from Da, known to occur in large amounts in soybeans (45). Algae may also be a source of isoflavonoids because a positive correlation was found with Da ($r = 0.56$; $P < 0.05$) and total isoflavonoids ($r = 0.45$; $0.05 < P < 0.10$, NS). Algae were suggested to contain factors protective against breast cancer (46).

Lignans and bioflavonoids are candidates for a role as cancer-protective agents (2, 14–16) and as steroid competitors for various enzymes (47). Enl inhibits the aromatase enzyme and competes with the natural substrate androstenedione for the binding site on the cytochrome P450 enzyme (H Adlercreutz, C Bannwart, LE Vickery, et al, unpublished observations, 1985). Phytoestrogens and lignans (48; H Adlercreutz, Y Mousavi, J Clark, et al, unpublished observation, 1987) show interaction with estrogen receptors and flavonoids have antiproliferative effects on the human-breast-carcinoma cell line ZR-75-1 (49). Genistein is a very specific inhibitor of the tyrosine-specific protein kinases (50–55) and platelet-activating-factor–stimulated platelet aggregation, phospholipase C, and tyrosine kinase activity (56). Tyrosine kinase is an important mediator of the effects of some biologically important growth factors such as epidermal growth factor, insulin, platelet-derived growth factor, and insulin-like growth factor on cells. The flavonoids and lignans bind to the type II estrogen-binding sites (15, 57), now also called the bioflavonoid receptor (47, 58), and may in this way regulate by inhibition cell growth and proliferation of hormone-dependent cancers (58). Enzymes metabolizing bioflavonoids and steroids show structurally close similarity (47), indicating that they have the same origin. Furthermore, the isoflavonoid coumestrol complements, as does estradiol, the topography of spaces between base pairs in unwound DNA and simultaneously hydrogen-bond phosphate moieties on opposite strands (59).

One of the most important biological effects of the lignans and isoflavonoids seems to be their stimulation of SHBG synthesis in the liver (2, 14, 16–18). A high SHBG concentration leads to decreased metabolic clearance rate for the sex hormones and lower biological activity. However, Japanese and British women were found to have the same SHBG total-binding capacity, even though Japanese women bound relatively more estradiol to SHBG. This was suggested to be a result of lower affinity of albumin for estradiol in these women (60). It is possible that the phytoestrogens in the high amounts occurring in Japanese women could compete with estradiol for the albumin-binding sites and in this way lead to relatively more binding to SHBG.

SHBG concentrations tend to be lower in breast-cancer patients, particularly in postmenopausal women, and this seems at least partly to be due to diet (15). SHBG-binding capacity was significantly smaller in postmenopausal but not in premenopausal Japanese subjects with breast cancer compared with Japanese control subjects (61), agreeing with our own more recent results in American postmenopausal (43) women. Finnish premenopausal women with breast cancer did not differ in this respect from omnivorous control subjects but they had lower SHBG than did lactovegetarian women (18). Diet seems to be a much more important risk factor for postmenopausal than for premenopausal breast cancer (15). Miso (Japanese soybean paste) (62) or powdered soybean chips (63) (both before and after denaturation of the protease inhibitors) showed a tendency to decrease mammary-tumor formation and growth rate in rat breast-cancer models and soybean diet also reduced breast-tumor incidence in irradiated rats (64). This agrees with the slower average growth rate of postmenopausal breast cancers in Japanese compared with caucasian women in Hawaii (65).

The high concentration of phytoestrogens in the urine of Japanese men could be protective with regard to prostate cancer. Both lignans and isoflavonoids have estrogenic effects in numerous biological systems and may, because of this property, inhibit development of prostatic cancer. It is well known that in Japan and some other Asian countries, despite the same incidence of latent small or noninfiltrative prostatic carcinomas as in Western societies, the mortality is low (25–27). The high exogenous phytoestrogen concentrations could inhibit the growth of the latent carcinomas, postponing their development and making it more likely that the subjects die from some other disease (theory proposed in 1985) (66). Furthermore, the inhibitory effect of genistein on tyrosine-specific protein kinases of certain growth-factor receptors could play an important role. Decreased risk of prostate cancer is seen in Seventh-day Adventist men (67) consuming much beans, lentils, and peas and some dried fruits (rich sources of bioflavonoids) and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which has some fiber-like effects in the gut) and tofu (68), supporting the view that these compounds are protective. Recently, Santti's group in Turku, Finland, in a collaborative study with us, observed that dietary soy prevented the development of precancerous changes in a neonatally estrogenized mouse used as a model for prostatic cancer (69), showing that dietary factors may already be important in the fetal and neonatal periods. This study and our observation of high phytoestrogen excretion in urine of children is important because they suggest that these compounds may change the endocrine milieu at the cellular level both in the neonatal period and in prepubertal and adolescent children. Thus, the results cited above and discussed more

extensively elsewhere (14, 15) speak for a role of the diphenols as cancer-protective substances.

It is concluded that Japanese subjects excrete very large amounts of isoflavonoids in urine, mainly genistein, daidzein, and equol, and that the lignan excretion is low. The high excretion of isoflavonoids in urine is related to the intake of soy products in the traditional Japanese diet.

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APPENDIX A

Additional experiments with a modification of the method

The method used in this study was modified further by including the determination of the plant lignan matairesinol [(3R-*trans*)-dihydro-3,4-*bis*[(4-hydroxy-3-methoxy-phenyl)methyl]-2(3H)-furanone)] (intraassay CV = 15.2% and interassay CV = 13.9%) and the isoflavonoid genistein (4',5,7-trihydroxyisoflavane) (intraassay CV = 4.5% and interassay CV = 11.6%) in the assay (1). Because further samples from the present study were not available and because of the recent great interest in genistein we used this new assay in nine other Japanese subjects (three men, three women, and three children) living in Kyoto and consuming a traditional Japanese diet before and during the 24-h urine collection.

TABLE 1A

Urinary excretion of lignans and isoflavonoid phytoestrogens (μmol/d) in nine Japanese subjects (six adults, three children) living in Kyoto and consuming traditional Japanese diet during the urine collection period

Subject, sex, age	Matairesinol	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Genistein	Total isoflavonoids	Total diphenols
1, M, 41 y	0.010	0.05	0.09	0.15	5.25	6.15	0.12	15.52	27.04	27.20
2, F, 33 y	0.003	2.44	0.15	2.59	3.11	0.01	0.98	4.48	8.58	11.17
3, M, 7 y	0.003	0.07	0.09	0.16	3.23	0.01	0.06	5.66	8.97	9.13
4, M, 6 y	0.006	2.24	0.68	2.93	2.15	0.85	0.51	3.41	6.93	9.85
5, M, 8 y	0.007	0.04	3.39	3.43	3.02	0.02	0.81	4.80	8.64	12.07
6, F, 42 y	0.006	3.25	0.25	3.50	2.20	0.16	1.17	3.55	7.07	10.58
7, M, 38 y	0.012	0.70	0.25	0.96	1.60	0.07	0.40	4.93	6.99	7.95
8, M, 26 y	0.019	1.94	0.18	2.13	3.38	9.16	0.23	7.99	20.76	22.89
9, F, 30 y	0.005	0.62	0.25	0.88	1.25	3.28	0.21	1.85	6.60	7.47
$\bar{x}$	0.010	1.26	0.59	1.86	2.8	2.19	0.50	5.80	11.29	13.15
Geometric $\bar{x}$	0.010	0.50	0.27	1.17	2.58	0.25	0.35	4.91	9.81	11.89

Table 1A shows the individual urinary lignan and isoflavonoid excretion in the additional three men, three women, and three children studied by the new modified procedure, including the results of assays for matairesinol and genistein.

Reference

1. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta 1991;199:263–78.

Use of a Mammalian ... a)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from *Trifolium pratense* L.[1]

D2

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ABSTRACT

Based on the epidemiological evidence for a relationship between consumption of certain foods and decreased cancer incidence in humans, an assay was developed to screen and fractionate plant extracts for chemopreventive potential. This assay measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene [B(a)P] in hamster embryo cell cultures. Screening of several plant extracts has generated a number of activity leads. The 95% ethyl alcohol extract of one of these actives, *Trifolium pratense* L. Leguminosae, red clover, significantly inhibited the metabolism of B(a)P and decreased the level of binding of B(a)P to DNA by 30 to 40%. Using activity-directed fractionation by solvent partitioning and then silica gel chromatography, a major active compound was isolated and identified as the isoflavone, biochanin A. The pure compound decreased the metabolism of B(a)P by 54% in comparison to control cultures and decreased B(a)P-DNA binding by 37 to 50% at a dose of 25 μg/mL. These studies demonstrate that the hydrocarbon metabolism assay can detect and guide the fractionation of potential anticarcinogens from plants. The ability of the isoflavone biochanin A to inhibit carcinogen activation in cells in culture suggests that *in vivo* studies of this compound as a potential chemopreventive agent are warranted.

INTRODUCTION

Humans are exposed to numerous carcinogens and mutagens daily, some avoidable (such as cigarette smoking) and some virtually unavoidable (diet, environmental pollution, oxygen radicals). The diet has been shown to have a profound effect on the incidence and location of various human cancers worldwide (1, 2), and epidemiological studies suggest that certain dietary components may help to prevent cancer induction. This prophylaxis has been termed cancer chemoprevention. Wattenberg (3) has demonstrated that such agents may inhibit cancer induction by a number of mechanisms. One of the more common mechanisms is through inducing alterations in the enzymatic activation or detoxification of carcinogens.

Although many biological assays have been used to examine the chemopreventive potential of various chemicals, there have been relatively few studies using activity-directed fractionation to isolate active compounds from plants. In addition, it is impractical to use *in vivo* models to guide these procedures. Loub *et al.* (4) used an activity-directed fractionation procedure based upon induction of aryl hydrocarbon hydroxylase activity in the liver and intestinal mucosa of Sprague-Dawley rats to isolate and identify several indoles from cruciferous vegetables. Kaweol and caffestol palmitates were isolated from green coffee beans (5) based upon an assay that measured the increase in glutathione S-transferase activity in liver and intestinal mucosa of mice. Practical assays for activity-directed fractionation of active plants must be rapid, sensitive, convenient, and capable of detecting alterations in carcinogen metabolism. In this paper, we describe the development and application of an assay that measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene, a widespread environmental carcinogen, in early passage cultures of Syrian hamster embryo cells (6). The chemical and analytical procedures developed for activity-directed fractionation of antineoplastic compounds from plants (7, 8) were adapted to the isolation and identification of potential anticarcinogens from food and food plants, such as red clover extracts, which significantly inhibited the metabolism of benzo(*a*)pyrene and binding of B(a)P[3] metabolites to DNA.

MATERIALS AND METHODS

Spectroscopy and Chromatography. ^{1}H NMR in deuteriochloroform was performed using a Varian XL-200, and ^{13}C NMR in deuteriochloroform was measured on a Chemagnetics A-200 spectrometer. EI and CI mass spectra were obtained on a Finnigan 4023 quadrupole mass spectrometer. High-resolution mass spectra were recorded on a Kratos MS 50. The IR spectrum was performed on a Beckman IR-33 using a KBr pellet. UV spectra were measured on a Beckman DU-7 in methyl alcohol using sodium methoxide, $AlCl_3$, HCl, and sodium acetate as UV shift reagents.

For flash column chromatography EM 9385 Silica Gel 60 was used for the adsorbent. Radial chromatography was performed on a Chromatotron Model 7924 using a 1-, 2-, or 4-mm rotor with EM 7749 Silica Gel 60 PF 254 as adsorbent. TLC plates were Merck 5714 Silica Gel 60 F_{254}.

Cell Culture Toxicity Assay. Hamster embryo cell cultures were prepared and grown as described previously (6). Tertiary cultures were plated in 60-mm plastic dishes (Falcon) (5×10^5 cells), and 24 h later the test compound was added at 10-fold dilutions from 500 μg/ml of medium to 0.05 μg/ml for 24 h. At that time the cultures which were approximately 70% confluent were examined microscopically and subjectively evaluated for the percentage of the cells dividing and the cell density. The highest noninhibitory dose was selected for metabolism studies.

B(a)P Metabolism Assay. Tertiary hamster embryo cell cultures (10^6 cells per 25-cm^2 flask, 3 flasks per group) were plated in 8 ml of medium containing 10% calf serum and refed with 8 ml of fresh medium after 48 h. Seventy-two h after plating, the cultures were treated with the test compound in DMSO or DMSO as a control and 30 min later [^{3}H]-B(a)P (1 μg/ml; specific radioactivity, 0.25 Ci/mmol) was added. Twenty-four h later medium was removed and stored at −20°C. Aliquots (0.2 ml) were extracted by a two-stage chloroform:methanol:water procedure (6, 9). The assay uses initial mixing with a vortex mixer in a single-phase system of chloroform:methanol:water (including the medium) (1:2:0.8) to ensure complete extraction of the lipophilic hydrocarbon and its metabolites followed by addition of 1 ml of chloroform

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[3] The abbreviations used are: B(a)P, benzo(*a*)pyrene; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; MS, mass spectrum; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; B(a)PDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; dGuo, deoxyguanosine.

for 10 min, the aqueous phase was re [illegible]

of chloroform to ensure complete extraction. The chloroform extracts were then pooled, and the radioactivity in the organic and aqueous-methanol phases was measured by liquid scintillation counting of 0.1-ml aliquots. This extraction procedure results in recovery of unmetabolized B(a)P and Phase I metabolites (dihydrodiols, quinones, and phenols) in the chloroform phase. The water-soluble metabolites including glucuronides and glutathione conjugates and multiple oxidation products are retained in the aqueous-methanol phase. Since the large majority of the metabolites formed from B(a)P in hamster embryo cells are water soluble (usually greater than 80%) (6), this assay provides a rapid measure of B(a)P metabolism.

BHA, a known inhibitor of carcinogenesis and B(a)P metabolism (10), was used to treat a positive control group in all assays at a concentration of 50 µg/ml of medium. The highest nontoxic dose of BHA was selected from multiple experiments using different hamster embryo cell preparations. Doses of 75, 65, 50, and 5 µg/ml of medium were tested, and the results show that 75 µg/ml were toxic and 65 µg/ml exhibited borderline toxicity, while 50 µg/ml showed a significant inhibition of B(a)P metabolism with no cell toxicity. The lowest dose, 5 µg/ml, produced no significant inhibition of B(a)P metabolism. Using BHA as a positive control gave us an indication of the health and viability of the cells in the culture assay for that particular experiment and helped eliminate false negatives.

Analysis of B(a)P Metabolites. The B(a)P metabolites in the organic phase were analyzed by HPLC on an Ultrasphere C_{18} column (25 cm × 4.6 mm) eluted with a methanol:water gradient as described previously (6). UV absorbing standards of authentic B(a)P metabolites (Chemical Repository, Division of Cancer Etiology, National Cancer Institute) were included in each HPLC analysis. The radioactivity was monitored with a Flo-one β flow monitor set to update every 30 s.

Binding of B(a)P to DNA. Tertiary hamster embryo cell cultures (5 × 10^7 cells) were plated in 175-cm^2 flasks containing 50 ml of minimal essential medium with 10% fetal bovine serum. After 2 days the cultures were refed with fresh medium and 24 h later with the test compound, or extract in DMSO was added. Five to 10 min later the cultures were treated with [^3H]B(a)P (1 µg/ml of medium, 0.5 mCi/flask). After 24 h of incubation at 37°C the cells were harvested, and DNA was isolated as described previously (11). The radioactivity in an aliquot was measured by liquid scintillation counting, the amount of DNA was determined by A_{260}, and these values were used to calculate the level of B(a)P metabolites bound to DNA.

After enzymatic degradation of the DNA to deoxyribonucleosides, the B(a)P:deoxyribonucleoside adducts were isolated by chromatography on Sep-Pak C_{18} cartridges and analyzed by HPLC on a 25-cm × 4.6-mm Ultrasphere C_8 reversed-phase column (11). The column was eluted at a flow rate of 1.0 ml/min with methanol:water (46:54) for 30 min, a linear gradient for 10 min (46:54 to 55:45) and at 55:45 for 24 min. Fifteen 1.0-ml fractions followed by 165 fractions (0.3 ml) were analyzed by scintillation counting.

Plant Extraction. Leaves, stems, and flowers of *Trifolium pratense* L. (red clover) were collected. A voucher specimen is on deposit in the biology herbarium of the Department of Biology, Purdue University. The fresh plant (918 g) was ground with 2 liters of 95% ethyl alcohol in a commercial size Waring blender for 5 min. The blended material was then allowed to stand for 30 min to complete the extraction. The material was then filtered through a Büchner funnel, and the filtrate was concentrated *in vacuo* to give 46.5 g of the 95% ethyl alcohol extract. An aliquot was dissolved in DMSO and submitted for testing. The 95% ethyl alcohol extract was found to be active and therefore was then further partitioned according to the scheme shown in Fig. 1. The testing data are shown in Table 1. All fractions were tested at the dose-response dose which was defined as the percentage of the 95% ethanol-extractable material that the fraction represented times the dose of 95% ethanol fraction used in the metabolism assay (in this case, 750 µg/ml of medium).

Isolation and Identification of Active Components. Aliquots from the solvent partition were submitted for testing. The active $CHCl_3$ fraction was subjected to silica gel flash column chromatography with hexane, [illegible] methyl alcohol. Nine fractions were collected, and aliquots were taken and submitted for testing. The column fraction which was active at the dose-response dose (Fraction 1D) (see Fig. 1) was further chromatographed by centrifugal silica gel TLC (Chromatotron) using a $CHCl_3$/methyl alcohol solvent gradient starting with 2% methyl alcohol in $CHCl_3$. The fractions which were collected were combined according to the presence of similar spots when analyzed by silica gel TLC developed in 2% methyl alcohol in $CHCl_3$. Based upon this, the samples were combined into seven fractions which were tested for their effects on B(a)P metabolism. The most active fraction (2D) was further separated on another silica gel Chromatotron plate developed in a $CHCl_3$/methyl alcohol gradient. Based upon TLC profiles eluants were combined into three fractions. The most active fraction (3B) contained a major component. Recrystallization of this fraction from aqueous methyl alcohol gave a crystalline material, m.p. 217–218°C. A sample of authentic biochanin A was purchased from Aldrich Chemical Co., m.p. 218–219°C. A mixed m.p. showed no depression. The UV and ^1H NMR data were identical to literature values (12), and the MS and ^{13}C NMR data were consistent with the published structure.

Examination of the interface fraction, which was active at 2× the dose-response dose, led to the isolation of additional biochanin A, along with an analogue, formononetin (see Fig. 3). Formononetin was inactive in the metabolism bioassay. Biochanin A represented about 30% of the interface fraction.

RESULTS

The results of bioassay-directed fractionation of the active ethyl alcohol extract of red clover are presented in Fig. 1 and Table 1. The ethyl alcohol extract was active at doses from 500 µg/ml to 1000 µg/ml; however, toxicity was detected at the highest dose (see Table 1). Further partitioning of the active ethyl alcohol extract was dose responded from 750 µg/ml. After partitioning between chloroform and water, the activity appeared in the chloroform extract. Examination of the interface which was active at twice the dose-response dose confirmed the presence of biochanin A. Chromatography of the chloroform fraction gave active column Fraction 1D. This fraction was carried through two separations on the Chromatotron to give in turn active Fractions 2D and 3B. Crystallization of Fraction 3B gave 30 mg of the active constituent, biochanin A. Fractions 1A, 1B, 2B, and 2F show activity at twice the dose-response dose and are under further investigation. The B(a)P metabolites present in the organic phase of the sample treated with red clover extract at 500 µg/ml were analyzed by HPLC, and the amount of the major primary B(a)P metabolites was determined (Fig. 2). The two major changes were a slight increase in the amount of 9-hydroxy-B(a)P and a major decrease in the amount of water-soluble metabolites in the extract-treated group. After β-glucuronidase treatment of the aqueous phase, the amount of 9-hydroxy- and 3-hydroxy-B(a)P in the red clover extract-treated group was reduced by 30% and 22%, respectively, when compared to DMSO controls. The water-soluble metabolites were also decreased by 18% in the red clover extract-treated cells. Thus the major effect of red clover extract was to inhibit the formation of B(a)P-phenol glucuronides.

The effect of the crude 95% ethyl alcohol extract on the binding of B(a)P to DNA was also examined (Table 2). At a dose of 250 µg/ml the extract inhibited B(a)P-DNA binding by 30% to 41% compared to controls in three separate experiments. Analysis of the B(a)P-DNA adducts present in enzyme-digested DNA samples by HPLC demonstrated that the extract inhibited the formation of both the *syn*- and *anti*-isomers of B(a)PDE. The *syn*-B(a)PDE-dGuo adducts decreased from 37% to 64% compared to controls, and the (+)-*anti*-B(a)PDE-dGuo adduct decreased from 48% to 75%.

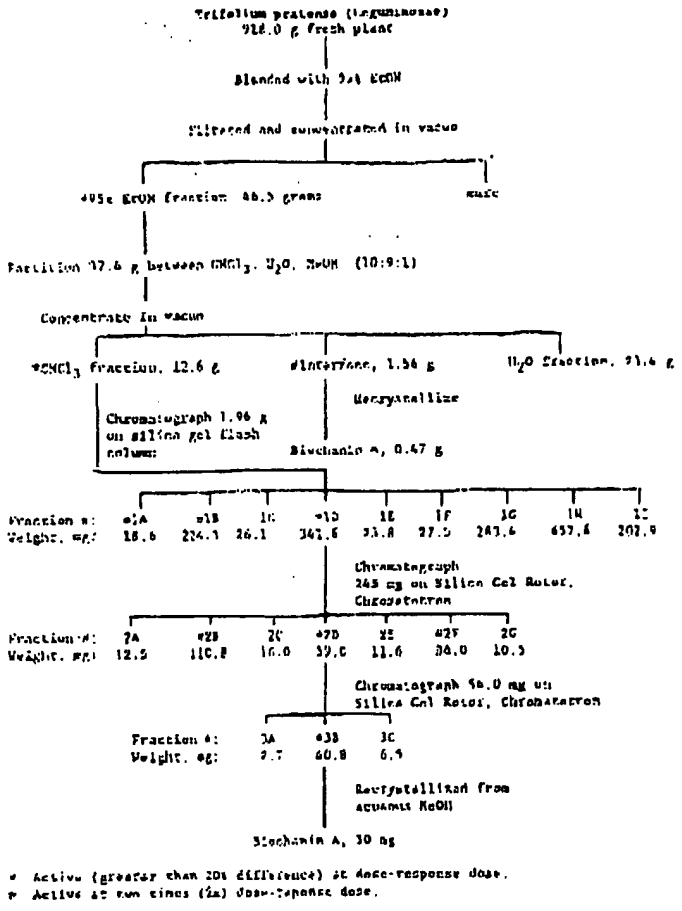

Fig. 1. Fractionation scheme for the 95% ethyl alcohol extract of fresh red clover leaves, flowers, and stems. *EtOH*, ethyl alcohol; *MeOH*, methyl alcohol.

* Active (greater than 20% difference) at dose-response dose.
** Active at two times (2x) dose-response dose.

The effect of biochanin A on the binding of B(a)P to DNA in hamster embryo cell cultures was also examined. Biochanin A caused a 54% decrease in B(a)P metabolism at 25 μg/ml. After exposure of cultures to 25 μg of biochanin A and 1 μg of [^{3}H]B(a)P per ml of medium for 24 h, biochanin A treatment reduced the amount of B(a)P bound per mg of DNA from 74.3 pmol in the control group to 35.1 pmol in the biochanin A group in one experiment and from 72.2 to 45.4 in a second experiment. Thus, biochanin A inhibited the binding of B(a)P to DNA to an extent similar to that obtained in the crude extract (Table 2).

DISCUSSION AND CONCLUSIONS

There are several bioassays which are under investigation for the detection of compounds suspected of having potential cancer chemopreventive activity. Antimutagenic activity in the form of an anti-Ames assay has been commonly used in the United States and Japan (13, 14). Mitscher *et al.* (15) used this bioassay to isolate and identify glabrene, a known isoflavene exhibiting antimutagenic activity. Nishino *et al.* (16) used the antitumor-promoting activity of glycyrrhetic acid against 7,12-dimethylbenz(a)anthracene and teleocidin as a model of cancer prevention. The decrease in formation of carcinogenic *N*-nitroso compounds produced by α-tocopherol and ascorbic acid was used as a criterion for chemoprevention by Narkus *et al.* (17) and Mervish (18). Sakiyama *et al.* (19) used the inhibition of transformation of the mouse 10T½ cell line induced by X-ray or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a model to show the anticarcinogenic effects of lipopolysaccharides and indomethacin. The induction of aryl hydrocarbon hydroxylase activity in liver and intestinal mucosa of Sprague-Dawley rats was used by Wattenberg *et al.* (3) to isolate and identify a group of indoles from cruciferous vegetables (20, 21). Another screen by Wattenberg *et al.* (5) used the induction of glutathione *S*-transferase activity, a major detoxification enzyme system, for a number of electrophiles, including many carcinogens, in mouse liver and intestinal mucosa to isolate a group of known diterpenes from green coffee beans.

The screening procedure described in this paper measures effects on the ability of hamster embryo cell cultures to metabolize the carcinogen B(a)P. Induction of inhibition of B(a)P metabolism of treated cultures by >20% as compared with control cultures was considered to be an active test. The altered pattern of metabolism was determined by HPLC analysis of the B(a)P metabolites formed, and the effects on binding of B(a)P to DNA are determined. Confirmed active extracts are then fractionated using the bioassay as a guide. Advantages of our method are that activity data can be generated within a few days after the extract or compound is tested, and a large number

The procedure used for analysis of B(a)P [illegible] is described in "Materials and Methods."

Fraction	Dose-response dose (μg/ml medium)	% of change from control	2X dose-response dose	% of change from control
95% Ethyl alcohol	500	−39.9 ± 6.2[a, b]		
	750	−40.4 ± 6.9[a, c]		
$CHCl_3$	203	−35.6 ± 5.7[a, c]	406	−65 ± 4.4[d]
Interface	25.2	−17.7 ± 7.3[e]	50.2	−33.8[d, c]
H_2O	561	−15.4 ± 1.4[e]		
1A	1.9	17.2 ± 5.4[b]	3.9	28.8[d, c]
1B	23.5	−5.9 ± 15.2[d]	46.6	−34.6 ± 4.6[d, c]
1C	3.7	8.7 ± 5.6[e]	7.4	−8.0[d]
1D	35.5	23.5 ± 4.0[b, c]	71.0	−50.2[d, c]
1E	2.5	3.25 ± 6.8[e]	5.0	−3.5[e]
1F	2.8	8.5 ± 0.8[e]	5.6	13.6[e]
1G	29.4	12.1 ± 7.3[e]	58.8	17.8[e]
1H	68.1	5.0 ± 9.2[e]	136.2	−5.5[e]
1I	21.0	−5.9 ± 11.4[e]	42.0	−11.7[e]
2A	1.8	−14.1[e]	3.6	4.8[e]
2B	16.1	−12.9[e]	32.2	−32.7[d, c]
2C	2.3	−13.8[e]	4.6	−12.9[e]
2D	8.6	−25.3[d, c]	17.2	−19.0[e]
2E	1.7	−13.0[e]	2.4	3.0[e]
2F	4.9	−14.6[e]	9.8	−23.0[d, c]
2G	1.5	−1.8[e]	3.0	
3A	0.4	−6.8[e]	0.8	
3B	6.4	−23.7[b, d]	12.8	−23.0 ± 22.9[c, f]
3C	1.0	5.93 ± 18.6[e]	2.0	8.8[e]
Biochanin A	4.7	−12.2[d]		
	9.5	−33.1[e]		
	19.0	−47.4[d]		
	23.6	−48.8[d]		

[a] Mean ± SD of 3 experiments.
[b] Active (greater than 20% difference) at dose-response dose.
[c] Average ± range of 2 experiments.
[d] One experiment.
[e] Active at 2X dose-response dose.
[f] Mean ± SD of 4 experiments.

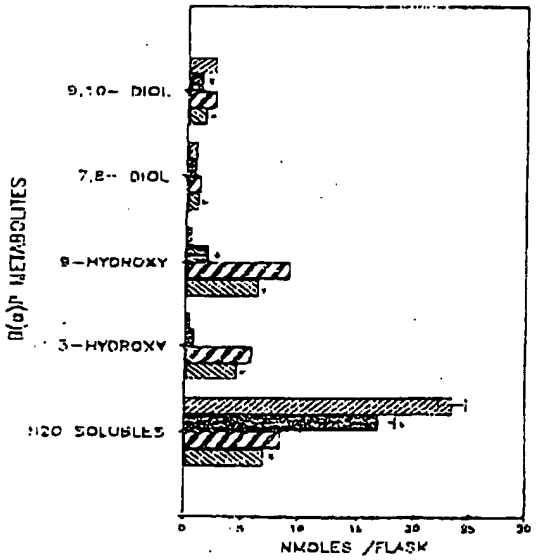

Fig. 2. The amount of B(a)P metabolites formed in hamster embryo cell cultures in the presence or absence of 500 μg/ml of red clover crude extract. The cultures were treated, and the medium samples used analyzed as described in "Materials and Methods." Medium samples were treated with β-glucuronidase prior to extraction to determine glucuronide conjugates. *Columns*, mean for 3 flasks per group; *bars*, SD. *, red clover extract-treated samples that differed significantly from the corresponding control (based upon Student's *t* test: $P < 0.01$); ▨, control; ▩, *T. pratense*/control (β-Glucuronidase); ▤, *T. pratense* (β-Glucuronidase).

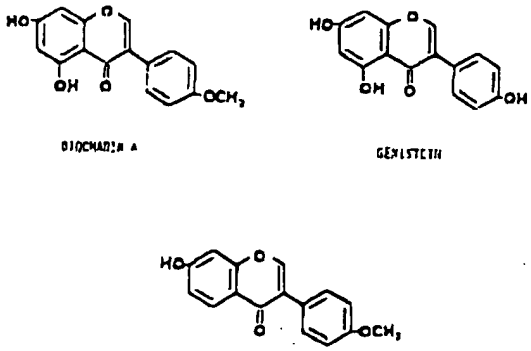

Fig. 3. Isoflavones isolated from red clover leaves and flowers (25, 26).

of different samples can be screened simultaneously. After pure active compounds are isolated and their effect on the metabolic activation of B(a)P is established, they will then be further tested using *in vivo* bioassays to determine their effect on tumor induction by various classes of carcinogens. These *in vivo* bioassays are essential for determining whether a compound acts as an anticarcinogen and against which classes of carcinogens it was active.

Thus far we have screened over 70 species and varieties of plants and vegetables comprising 27 families. One of the first plant extracts demonstrated to produce reproducible inhibition of B(a)P metabolism in the hamster cell culture assay was that prepared from red clover. Based upon inhibition of B(a)P metabolism the crude red clover extract was fractionated, and

Table 4 B(a)P-DNA binding in hamster cfa

Hamster embryo cell cultures were exposed to the 95% ethyl alcohol extract of red clover at a dose of 250 μg/ml of medium, and 10 min later 1.0 μg of [^{3}H] B(a)P per ml of medium was added. After 24 h, the medium was removed, and a sample was analyzed by chloroform:methanol extraction as described in "Materials and Methods." The percentage of radioactivity in the water phase is reported as the percentage of water-soluble B(a)P metabolites. The DNA was isolated from the cells, and the level of binding of B(a)P was measured. The DNA was digested to deoxyribonucleosides, and the amount of the major B(a)P-DNA adducts was determined by HPLC.

	Experiment 1		Experiment 2		Experiment 3	
	Control	Test extract	Control	Test extract	Control	Test extract
% of water-soluble B(a)P metabolites	48.6	40.5	30.8	15.4	35.6	27.5
Binding of B(a)P to DNA; total level of binding (pmol/mg DNA)	67.0	42.7	51.2	30.0	25.0	15.8
(+)-*anti*-B(a)PDE-dGuo adduct (pmol/mg)	12.7	4.9	11.1	2.7	5.5	2.9
syn-B(a)PDE-dGuo adduct (pmol/mg)	8.4	3.0	8.1	3.7	5.0	3.3

a pure active compound, biochanin A, was isolated which produced an inhibition of B(a)P metabolism of 30 to 50% at 9.5 to 23.6 μg/ml compared to DMSO controls. Exposure of hamster embryo cell cultures to biochanin A at a dose of 25 μg/ml of medium resulted in a 37 to 50% inhibition in the binding of B(a)P to DNA. This compound appears to be one of the major components responsible for the inhibition of B(a)P-DNA interactions by the red clover extract. The strong correlation between the binding of aromatic hydrocarbons to DNA and their carcinogenic activity suggests that biochanin A is a good candidate for further testing to measure inhibition of tumor induction by hydrocarbons in animals.

Several flavonoids have been shown to possess anticarcinogenic activity (3). 7,8-Benzoflavone, a synthetic flavonoid, is an inhibitor of microsomal mixed-function oxidases and inhibits the metabolism, binding to DNA, and tumorigenesis of 7,12-dimethylbenz(*a*)anthracene in mouse skin (22). This same flavonoid also inhibits the metabolism of B(a)P in rat hepatic microsomes that have been induced with 3-methylcholanthrene (23). Huang *et al.* (24) examined 28 flavonoids for their effect on mutagenicity of anti-B(a)PDE in *Salmonella* and found that 8 had significant antimutagenic activity. Interestingly one of the flavonoids found to be inactive (50% inhibitory dose > 100) was genistein, an isoflavone related to biochanin A (see Fig. 3) and a minor constituent of red clover (25, 26). Since the compound tested [B(a)PDE] was an ultimate mutagenic metabolites of B(a)P, that assay would not be expected to detect compounds that alter metabolic activation of B(a)P. Thus, various types of short-term assays may be anticipated to detect anticarcinogens that work by different mechanisms. In view of the requirement of the majority of classes of chemical carcinogens for metabolic activation and the ability of the metabolism assay to measure changes in enzymes both involved in activation as well as detoxification, the hamster cell assay should be capable of detecting modifiers of carcinogen metabolism that act by a number of mechanisms. The results demonstrate that the effects of test compounds on B(a)P metabolism and DNA binding in hamster embryo cell cultures can be used to screen and isolate pure compounds with potential anticarcinogenic activity from plants and other natural products.

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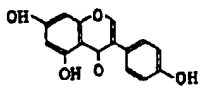
(54) CARCINOSTATIC AGENT

(57) Abstract:

PURPOSE: To provide a carcinostatic agent containing 5,7,4'-trihydroxyisoflavone as an active component and having tumor cell proliferation inhibiting activity and DNA-synthesis inhibiting activity.

CONSTITUTION: The objective agent contains 5,7,4'-trihydroxyisoflavone (general name: genistein) as an active component. Genistein is a compound separated from a certain kind of clover (Trifolium subterraneum L.) and is known to have week estrogen activity. It has been found newly that the compound is effective to inhibit the proliferation of tumor cell, the synthesis of DNA and the activity of tyrosine-specific phosphorylase. Coupled with the low acute toxicity, the compound is useful as a carcinostatic agent for the remedy of human and animal cancer, the remedy for diseases caused by the metastasis of cancer and the prevention of relapse of cancer. It is applied at a rate of usually 200W1,000mg daily in 1W4 divided doses.



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SPECIFICATIONS

1. Title of the Invention

Carcinostatic Agent

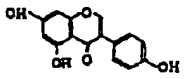
2. Claims

A carcinostatic agent whose active principle is 5,7,4'-trihydroxyisoflavone (genistein)

3. Detailed Description of the Invention

Applicable field of industry

This invention relates to a carcinostatic agent whose active principle is 5,7,4'-trihydroxyisoflavone (commonly known as genistein), and which is represented by the formula



Prior art

Genistein is a known compound, recorded in 1951 on page 3447 of the *Journal of the Chemical Society*. According to this article, genistein is a compound separated from a type of clover (*Trifolium subterraneum L.*), and is reported to have a weak oestrogen effect. However, absolutely no carcinostatic action is reported.

Action and effects of the present invention

The inventors of the present invention recognized a substance in the fermentation products of microorganisms belonging to the *Pseudomonas* genus isolated from soil as having

carcinostatic action and, as a result of further research into the substance, determined that the substance was genistein, and perfected the present invention.

The following describes the carcinostatic properties and toxicity of the compound of the present invention.

← Tumour cell propagation inhibiting action and DNA synthesis inhibiting action

The carcinostatic properties of genistein were investigated through the following experiments on the inhibition of the propagation and inhibition of the synthesis of DNA in the following experimental tumour cells.

(a) Tests on inhibiting propagation of rat cells transformed with Rous' sarcoma virus (RSV-3Y1)
(b) Tests on inhibiting propagation of human epidermal carcinoma cells (A431 cells)
(c) Tests on inhibiting propagation of rat cells transformed with SV40 virus (SV 40-3Y1 cells)
(d) Tests on inhibiting synthesis of DNA in mouse mast cell carcinomas (P815 cells)
(e) Tests on inhibiting synthesis of DNA in mouse thymuses (EL-4 cells)

Test methods and results of tests

The tests (a), (b) and (c) above were performed in the following manner.

The RSV-3Y1 cells (a), A341 cells (b) and SV40-3Y1 cells (c) were cultured in *Dulbecco MEM* (manufactured by Nippon Suisan KK) containing 2% calf embryo serum (manufactured by Gibco) and varying concentrations of genistein. Four levels of genistein concentration were employed: none added, 1 μg/ml, 3 μg/ml and 10 μg/ml. The numbers of live cells per dish were counted by means of Trypan Blue after the first, second, third and fourth days. The results are set out in Figure 1 (a) to (b).

As can be seen from Figure 1, genistein was found to have the effect of arresting cell propagation at levels of addition of from 1 μg/ml to 3 μg/ml, and the effect of inhibiting cell propagation was very strong at a level of addition of 10 μg/ml.

The methods of tests (d) and (e) above were as follows.

P815 cells (d) and EL-4 cells (e) were suspended in RPMI 1640 culture (manufactured by Nippon Suisan KK) to which 2% 56° C 30 minute inactivated calf embryo serum (manufactured by Flow Laboratories) and 80 μg/ml gentamycin (Manufactured by Essex Japan) KK) had been added, and a final cell count of 2 x 10^5 cells/ml was produced. 200 μl per well of the cell suspension solution was placed in a 96 well flat-based microplate (manufactured by Sumitomo Bakelite), and genistein was added in the following concentrations: none, 1 μg/ml, 3 mg/ml and 10 μg/ml. The plate was cultured for 24 hours in a 37° C 5% CO_2 culture system, whereupon 0.1 μCi/well of [^{3}H] Thymidine (manufactured by Amersham (Japan) KK) was added, and the solutions were cultured for a further 18 hours. The cells in each well were collected by glass fibre filter (Whatman GF/C) , and the filters were dried and placed in scintillation vials, toluene scintillator was added, and the uptake by [^{3}H] Thymidine was measured by means of liquid scintillation counter. The results are set out in Figure 2.

As can be seen from Figure 2, when 3 μg/ml of genistein was present in the culture solution, the uptake of Thymidine by the P815 cells was restricted to approximately 50%, while at a level of 10 μg/ml, the uptake of Thymidine by the P815 and also EL-4 cells was completely inhibited.

↑ Inhibiting action against tyrosine-specific phosphorylase

The inhibiting action of genistein against various enzymes was measured in relation to the following three types of tyrosine-specific protein kinase (a to c), two types of serine and threonine protein kinase (d and e) and other enzymes (f to h).

(a) Tyrosine-specific phosphorylase derived from mouse carcinoma virus (Src gene $pp60^{src}$)

(b) Human epidermal carcinoma cell propagation factor receptor (EGF receptor, A431 cell) tyrosine-specific phosphorylase

(c) Tyrosine-specific phosphorylase derived from cat carcinoma virus (fes gene, $pp110^{fes}$)

(d) c-AMP dependent protein kinase

(e) Phosphorylase kinase

(f) Phosphodiesterase

(g) Na^+, K^+-ATPase

(h) 5'-nucleotidase

Of these, (a) to (c) related to tyrosine-specific phosphorylase derived from carcinoma genes, and (d) and (e) to serine and threonine protein kinase.

The method of measurement of the inhibiting action of genistein on the enzyme activity of these, and the results of such measurements, are shown below.

Method of measurement

(a) Method of measurement of activity of tyrosine-specific phosphorylase derived from Rous' sarcoma virus (Src gene $pp60^{src}$) (see M.S. Collet and R.L. Elligson: *Proceedings of the National Academy of Sciences of the USA*, vol.75 pp2021-2024 (1978))

3Y1 cells (fibroblasts derived from rat embryo kidney) transformed by Rous' sarcoma virus (RSV) are grown, and after washing with RIPA buffer (0.5% NP40, 0.1% sodium deoxycholate, 50 mM Tris-HCl pH 7.2, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.15M NaCl) is added, and the cells are solubilized by being allowed to stand

for 30 minutes at 0° C. This is centrifuged for 20 minutes at 100,000 x g and is then inoculated with RSV, antiserum obtained from carcinoma-infected rabbits is added and the mixture is incubated for from 30 minutes to 1 hour at 0° C and the $pp60^{src}$ and antibodies are reacted together. The immune complex is concentrated by mixing with protein A-Sepharose-4B (manufactured by Pharmacia) and is then washed in RIPA buffer. The $pp60^{src}$-antibody- protein A-Sepharose-4B complex so formed reacts for 5 minutes at 30° C in 20 mM Pipes-NaOH pH 7.2, 5 mM $MgCl_2$, 1 mM DTT and 10 μm [γ-^{32}P] ATP (2 mCi/mmol), the protein kinase reaction is performed, whereupon a reaction halting solution containing SDS is added, the mixture is boiled for 3 minutes and the reaction is halted. The reaction solution is subjected to electrophoresis with 8% SDS-polyacrylamide gel, and after autoradiography, the radiation from the $pp60^{src}$ is measured by means of a liquid scintillation counter, and the phosphorylation reaction is quantified.

(b) Method of measurement of activity of tyrosine-specific phosphorylase from human epidermal carcinoma cell proliferation factor receptor (EGF receptor, A431 cell) (See S. Kornin G. Carpenter, and L. King: *Journal of Biological Chemistry*, vol. 255, pp.4834-3842 (1980))

Cell membranes prepared from human epidermal carcinoma cells (A431 cells) that are known to contain large numbers of EGF receptors are used as the enzyme source. A reaction solution containing genistein, 20 mM Pipes-NaOH pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 10μM (γ-^{32}P) ATP (2mCi/mmol) and A431 cell membrane (protein content 10 μg) is allowed to react together in 50 μl for 5 minutes, whereupon the reaction is halted, whereupon the reaction solution is subjected to electrophoresis with 8% SDS-polyacrylamide gel, and after analysis by autoradiography, the EGF receptors of molecular weight of 170,000 were examined for the presence of phosphorylation. The EGF receptors were further isolated, and the radiation was measured by liquid scintillation counter, and the extent of phosphorylation was measured.

- Method of preparation of cell membranes from A431 cells

  A431 cells propagated in *Dulbecco* MEM (manufactured by Nippon Suisan KK) containing 7% calf embryo serum (manufactured by Gibco) were collected, and cell membrane follicles were prepared by the method of Rowen et al. (See Stanley Rowen, Hiroshi Ushiro, Krista Stosiek and Michael Cingaz: *Journal of Biological Chemistry*, vol. 257, pp.1523-1531 (1982)).

(c) Method of measurement of activity of tyrosine-specific phosphorylase from cat sarcoma virus (fes gene, $pp110^{fes}$) (See R.A. Feldman, T. Hanafusa and H. Hanafusa: *Cell*, vol. 22, pp.757-765 (1980))

Rat 3Y1 cells transformed with cat sarcoma virus and these cells were inoculated, and serum from cancer-bearing Fisher rats was used, and the protein kinase of immune precipitated $pp110^{fes}$ was measured after the same manner as for $pp60^{src}$.

(d) Method of measurement of activity of c-AMP-dependent protein kinase

c-AMP-dependent protein kinase prepared from rabbit muscle (protein content 4 µg) (manufactured by Sigma) reacts for 5 minutes at 30° C in 50 µl of a reaction solution containing 50 mM Hepes-NaOH pH 7.5, 10 mM $MgCl_2$, 4 µM [γ-^{32}P] ATP (2 mCi/mmol), 6 mg/ml histone type IIA (manufactured by Sigma), 10 µm c-AMP and genistein. This was spotted onto 2 x 2 cm Whatman filter paper P81, the filter paper was rinsed four times for 5 minutes on each occasion in 50 mM NaCl, and then was rinsed again with acetone, and the radiation was measured by means of a liquid scintillation counter.

(e) Method of measurement of phosphorylase-kinase activity

40 mM tris-HCl pH 7.4, 100 µm $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 10 µM [γ-^{32}P] ATP (2 mCi/mmol), 10 µg phosphorylase-b (manufactured by Sigma), rabbit muscle phosphorylase kinase (protein content 2 µg) (manufactured by Sigma) and genistein were reacted together in a 50 µl reaction solution for 5 minutes at 30° C, whereupon a reaction halt solution containing SDS was added, and the solution was boiled for 2 minutes at 100° C to halt the reaction. The phosphorylation of the phosphorylase-b was measured by 8% SDS-polyacrylamide gel electrophoresis – autoradiography of the reaction solution, followed by measurement of the separated phosphorylase-b by means of a liquid scintillation counter.

(f) Measurement of the activity of phosphodiesterase

50 mM tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.8 mM EDTA, 0.02 mM DTT, 5 mM c-AMP (manufactured by Sigma), cow heart phosphodiesterase (protein content 10 µg) (manufactured by Sigma) and genistein in 50 µl in a reaction solution were reacted for 30 minutes at 37° C.

50 µl of 10% TCA was added and the reaction was halted, the solution was centrifuged for 10 minutes at 5,000 rpm and 90 µl of the supernatant so derived was measured for phosphorus. The phosphorus colour reaction was measured by 660 nm absorbance after the addition to the supernatant solution of 3 µl of 1% *Triton X-100*, 350 µl of distilled water and 50 µl of 5N aqueous sulphuric acid containing 2.5% ammonium molybdate and allowing to stand for 20 minutes.

(g) Measurement of the activity of Na^+, K^+ Tpase

Na^+, K^+ Tpase was prepared by the method of Kawamura et al. (see Kawamura, Ota and Nagano: *Journal of Biochemistry*, vol. 87, pp.1327-1333 (1980)): The outer medulla of dog kidney was ground in a buffer containing 50 mM imidazole pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 0.1 mM ATP by polytron (manufactured by Kinematica) and then was ultracentrifuged to provide a microsome fraction that was extracted by means of SDS.

(h) Measurement of the activity of 5'-nucleotidase

A reaction solution containing 55 mM tris-HCl pH 8.5, 5.5 mM $MgCl_2$, 1.1 mM ATP, 10 mM potassium sodium tartrate, 5'-nucleotidase (snake venom) (manufactured by Sigma) and genistein was reacted in 50 µl for 3 minutes at 37° C, and the phosphorus levels of the reaction product were measured in the same manner as for phosphodiesterase.

Results

Inhibiting action of genistein in relation to various enzymes

Enzyme	ID_{50} (µg/ml)
(a) $pp60^{src}$ protein kinase	0.8
(b) EGF receptor protein kinase	0.7
(c) $pp110^{fes}$ protein kinase	6.5
(d) c-AMP dependent protein	>100

kinase	
(e) Phosphorylase kinase	> 100
(f) Phosphodiesterase	> 100
(g) Na^+, K^+-ATPase	> 100
(h) 5'-nucleotidase	> 100

ID_{50}: Level at which 50% inhibition occurs

As is clear from the above results, genistein has a specifically inhibiting action against tyrosine-specific phosphorylase derived from carcinoma genes.

Tyrosine-specific phosphorylase is believed to contribute to the propagation of carcinoma cells, and hence the recognition of a specific inhibiting effect against the action of this enzyme is the background to the carcinostatic effect of genistein.

→ C57BL/6 strain mice were injected in the abdominal cavity with genistein, and the acute toxicity of the genistein was examined. LD_{50} was found to be not less than 500 mg/kg.

In view of the above results of tests on its inhibiting effect on the propagation of tumour cells, its inhibiting effect on DNA, and its inhibiting effect on tyrosine-specific phosphorylase, genistein has an excellent carcinostatic effect, and moreover possesses a low toxicity, and thus is of value as a carcinostatic in the treatment of carcinomas in humans and animals, in the treatment of symptoms associated with metastasis of carcinomas, and in the prevention of relapse in carcinoma cases.

The clinical dosage of genistein is from 200 mg to 1000 mg of the active component per adult per day, administered in from one to four doses. The amount administered may be adjusted appropriately according to the individual circumstances of the patient, such as condition and age and so forth.

Genistein may be administered in isolation or in combination with other chemical treatment agents or immunological agents. Chemical treatment agents that may be employed in combination with genistein include cyclophosphamide, vinblastine, vincristine, adriamycin, 6-mercaptopurine, 5-fluorouracil, mytomycin C, pleomycin,

aclasinomycin, neocarzinostatin, cytosine arabinomide, actinomycin D, and nitrosourea and so forth. Immunological agents that may be employed in conjunction with genistein include for example creatine, BCG, [illegible], lentinan, interferon and interleukin and so forth. When genistein is employed in conjunction with other pharmaceuticals, the dosage of genistein is appropriately 1 of genistein to between 0.001 and 10 times the pharmaceutical employed in conjunction.

The dose of genistein may be prepared in form for oral administration (tablet, capsule or liquid) or non-oral administration (for rectal administration, inoculation or pellet). Such dose of genistein may be prepared as a combination with any commonly employed carrier or vehicle blended in the normal manner. Any generally employed carrier or vehicle may be employed, such as for example, in the case of tablets, water, fructose, lactose, gum arabic, gelatine, mannitol, starch paste, magnesium trisilicate, milk, maize starch, colloidal silica, potato starch or urea and the like. In liquid form, an aqueous or oleaginous suspension, solution, syrup, or elixir may be employed, and thee are prepared in the normal manner. For rectal administration, the genistein may be supplied as a suppository, and the base may be any normally employed base such as for example, polyethylene glycol, lanolin, cocoa fat, or *Witefzol*® (manufactured by Dynamit-Nobel) and so forth.

4. Simplified description of the drawings

(1) Figure 1 (a), (b) and (c) show the inhibiting effects of genistein on the propagation of RSV-3Y1 cells, A431 cells and SV40-3Y1 cells.

(2) Figure 2 shows the inhibiting effects of genistein on DNA synthesis in P 815 cells and EL-4 cells.

Figure 1 (a)

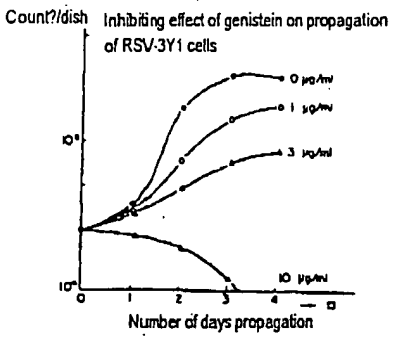


Figure 1 (b)

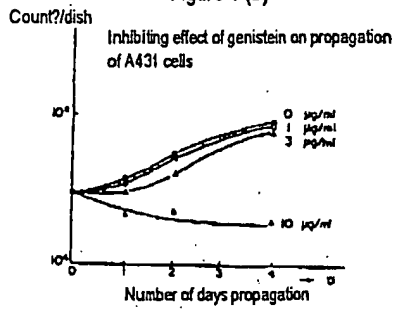
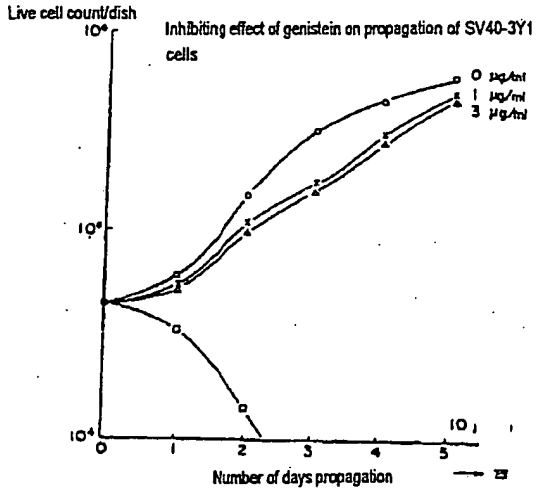


Figure 1 (c)



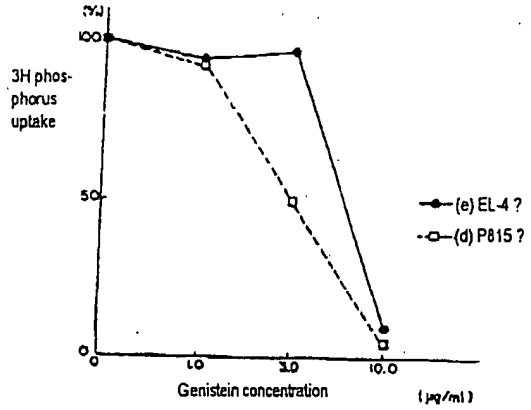

(%)
100
50
0
3H phos-
phorus
uptake
0
1.0
3.0
10.0
Genistein concentration
(μg/ml)
(e) EL-4 ?
(d) P815 ?

Figure 2

Amendment (Voluntary)

May 23 1985

Mr Manabu Shiga
Director, Patent Agency

1. Statement of Matter
Patent Application No. 89770 of 1985

2. Title of Invention
Carcinostatic Agent

3. Person making Amendment
Standing in matter: Patent Applicant
Name: Yamanouchi Pharmaceutical Co. Ltd
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Representative Shigeo Morioka (and another)

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5. Subject of Amendment
Detailed Description of Invention within Specifications

6. Contents of Amendment
See attached

(1) [English] Page 2 line 35 '(a) to (b)' changed to '(a) to (c)'

(2) [English] Page 3 line 19 'Src' changed to 'src'

(3) [English] Page 3 line 39 'Src' changed to 'src'

(4) [English] Page 4 line 4-5 'reacts' changed to 'is reacted'

(5) [Not relevant in English translation]

(6) [Not relevant in English translation]

(7) [Not relevant in English translation]

(8) [Not relevant in English translation]

(9) [English] Page 5 line 4 'reacts' changed to 'is reacted'; [Not relevant in English translation]

(10) [Not relevant in English translation]

(11) [Not relevant in English translation]

(12) [Not relevant in English translation]

(13) [English] Page 6 line 5 '3 minutes' changed to '30 minutes'

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(54) PRODUCTION OF ISOFLAVON COMPOUND

(57) Abstract:

PURPOSE: To inexpensively obtain a large amount of aglycones from an extracted solution or ground substance of soybeans, by heating soybeans or ground soybeans at a specific temperature in immersion, grinding and/or enzyme reaction process to maximize β-glucosidase activity in soybeans.

CONSTITUTION: In producing an isoflavon compound having estrogen action, antioxidation action, antihemolytic action, antilipemic action, cholesterol-lowering action and carcinostatic action from an extracted solution of soybeans or a ground material thereof, soybeans or the ground material is heated to 45-55°C in one process of immersion process, grinding process and enzyme reaction process after grinding to maximize β-glucosidase in the soybeans to give an isoflavon compound containing a large amount of aglycones such as daizein or genistein which is a main substance of medicinal effects such as carcinostatic action among isoflavon compounds and has extremely high utility.

19 JAPAN PATENT AGENCY (JP)
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51 Int Cl.[4]	Identification mark	Internal Agency Number
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C 07 H 17/07		7417-4C

43 Publication date: October 16 1989

Examination: Not requested Number of Inventions: 1 (Total 4 pages)

54 Title of Invention: Method of Manufacturing Isoflavone Compounds

21 Patent Application Number 63-83185

22 Date of Application: April 6 1988

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SPECIFICATIONS

1. Title of the Invention

Method of Manufacturing Isoflavone Compounds

2. Claims

A method of manufacturing isoflavones, such method characterised in that, when isoflavone compounds are manufactured from soybean extract solution or soybean meal, the soybeans or soybean meal is heated to between 45°C and 55°C during any one, or a plurality of, the soaking process, the grinding process, or the enzyme reaction process after the grinding process, in such a manner as to maximise the β-glucosidase activity in the soybeans.

3. Detailed Description of the Invention

Applicable field of industry

The present invention relates to a method of manufacturing isoflavone compounds and more particularly, isoflavone compounds that are rich in aglycones, from soybeans.

Prior art, and deficiencies thereof

Soybeans contain isoflavone compounds such as daidzin, clycitin, genistin, daidzein and genistein and so forth, and these are known to have such physiologically active effects as oestrogen effects, antioxidant action, antihaemolytic action, antibacterial action, anti-lipaemic action, and anti-cholesterol effects and so forth, and moreover, such isoflavone compounds have been recognised recently as possessing such cancer-controlling effects as inducing the differentiation of cancer cells and preventing the cancer genes and so forth, such that the value of such isoflavone compounds has received great attention.

Such cancer-controlling and other pharmaceutical effects of the isoflavone compounds are not due to glycosides, but principally to such aglycones as daidzein and genistein and the like.

JP 62-126186 for example reveals a method of deriving isoflavone compounds from soybean extract solution, but because at least 95% of the isoflavones present in soybeans are present as glycosides, the isoflavone compounds derived by this method are principally glycosides, and only very small amounts of the aglycones are produced.

Means employed in order to overcome such deficiencies

The inventors of the present invention investigated methods of deriving at low cost and in large volumes the aglycones that are the extremely valuable portion of the isoflavone compounds, and discovered that the action of β-glucosidase in soybeans readily breaks down the sugar bonds in the isoflavones in soybeans and converts such isoflavones to aglycones, and that such conversion reaches a maximum at a temperature of 50° C and a pH of 6.3.

The present invention was developed from this discovery, and is a method of manufacturing isoflavones, such method characterised in that, when isoflavone compounds are manufactured from soybean extract solution or soybean meal, the soybeans or soybean meal is heated to between 45° C and 55° C during any one, or a plurality of, the soaking process, the grinding process, or the enzyme reaction process after the grinding process, in such a manner as to maximise the β-glucosidase activity in the soybeans.

The following is a more detailed description of the present invention.

The soybeans that form the raw material may be in any form, provided only that the enzymes therein have not been deactivated, and such forms as minimally denatured de-fatted soybeans, soybean meal, shelled soybeans and whole soybeans and so forth may be employed.

In order to manufacture the isoflavone compounds from the extract solution from such soybeans, the soybeans are soaked in from five to ten times as much warm water at a temperature of from 45° C to 55° C, and the soaking water forms the extract solution which is the raw material for refining, while because the proportion of daidzein in such soaking water

is approximately 10% higher than that in soybeans, such soaking water forms an excellent raw material for refining.

Moreover, when soybean meal is employed for the manufacture of the isoflavones, soybeans that have been soaked in the manner described above may be ground together with the soaking water, or the soybeans may be soaked in water at room temperature and the soaked soybeans may then be ground at between 45° C and 55° C, or the soybeans may be heated to between 45° C and 55° C after grinding, in order to bring on the enzyme reaction, but preferably the soaking is conducted at from 45° C to 55° C and the beans are ground together with the soaking water at from 45° C to 55° C, with the meal so obtained being held at a temperature of from 45° C to 55° C for several hours. In this manner, the proportion of the isoflavones in the soybeans is greatly increased.

The soybean extract solution or soybean meal obtained in this manner is employed as the raw material for refining, but there are two methods of refining the isoflavone compounds.

One method involves refining by solvents. In this method, the raw materials for refining, namely, the soybean soaking water or the soybean meal, is powdered by drying in hot air or by freeze drying and the like, the powder so obtained is de-fatted by means of n-hexane or petroleum ether and the residue is dried and then extracted, whereby isoflavone aglycones only are obtained.

In the second method, the isoflavone compounds that are contained in the powdered raw materials for refining are reflux extracted by means of hydrated alcohol, the extract solution so obtained is concentrated and dried by known art, the residue is dissolved in a small amount of hydrated alcohol and adsorbed to a reverse phase type resin such as for example YMC-GEL ODS-A Type 60-01 (manufactured by Yamamura Kagaku Kenkyujo KK) or *Diaion HP-20* (manufactured by Mitsubishi Chemical Ltd) and the like, whereupon the resin is thoroughly rinsed with water, the phenolic acid is eluted with 20% hydrated alcohol, and isoflavone compounds rich in aglycones are separated and produced by means of 80% hydrated alcohol.

Moreover, the aglycone fraction only may be obtained by flushing the glycoside fraction with approximately 40% hydrated alcohol and elution with 80% hydrated alcohol.

The reverse phase resins employed in this case may be readily rinsed and regenerated with organic solvents such as for example alcohols or acetones and the like, and may then be reused.

Moreover, the raw materials for refining may be refined by direct adsorption to the resin, without the raw materials being powdered. Soaking water may be brought into contact with the reverse phase resins without further processing, or meal may be filtered by known art and the filtrate, or supernatant produced by centrifugal separation, may be brought into contact with the reverse phase resins.

The following describes examples of experiments relating to the present invention, and describes the effects thereof.

Experiment 1

Shelled soybeans were soaked for 6 hours in five times their own volume of water at between 20° C and 80° C, the soaked soybeans and the soaking water were cooled directly and frozen. The soaked soybeans and soaking water were dried in a freeze dryer and then powdered, a fixed amount was reflux extracted with 80% methanol, and a fixed amount of the measured material was analysed by high-speed liquid chromatography (Waters Model 209 D).

The results are set out in Table 1.

Table 1

Soaking temperature	Proportion of aglycones (%)			
	Soaked soybeans + soaking water		Soaking water	
	Genistein*	Daidzein**	Genistein*	Daidzein**
20 (° C)	3	5	-	-
30	8	8	-	-
40	14	16	20	36
45	18	24	23	39
50	23	29	24	40
55	21	26	33	45
60	12	15	18	36
70	6	7	-	-
80	4	4	-	-

$$* \frac{Genistein(mg)}{Genistein(mg)+Genistin(mg)} \times 100$$

$$** \frac{Daidzein(mg)}{Daidzein(mg)+Daidzin(mg)} \times 100$$

Experiment 2

Shelled soybeans were soaked for 2 hours in five times their own volume of warm water at 50° C that had been adjusted to a pH of between 5.5 and 11; the soaking water was acidified, and immediately filtered with a 0.45 μm filter, and analysed by high-speed liquid chromatography, in the same manner as for Experiment 1.

The results are set out in Table 2.

Table 2

Soaking water pH	Proportion of aglycones (%)	
	Genistein	Daidzein
5.5	33	42
6.3	39	43
6.9	35	39
8.0	33	35
9.0	19	23
10.0	17	20
11.0	15	16

As is evident from the results of Experiments 1 and 2, the maximum conversion to isoflavone aglycones is achieved at a soaking temperature of between 45° C and 55° C and a soaking water pH of 6.3.

Practical Embodiments

The following describes practical embodiments of the present invention.

Practical Embodiment 1

5 kg of shelled soybeans are soaked for 2 hours in 25 l of warm water that is maintained at 50° C.

Next, the soaking water is concentrated in an evaporator and dried, to yield 450 g of refining raw material. This is de-fatted by means of n-hexane, using a Soxhlet's extractor. The residue is then dried thoroughly and extracted by means of ethyl ether to yield 0.5 g of isoflavone aglycones.

Practical Embodiment 2

Shelled and soybeans that have been soaked as in Practical Embodiment 1 are ground together with their soaking water at 50° C, allowed to stand for 1 hour, and are then dried in a freeze dryer and powdered, to yield 4.1 kg of refining raw material. This is de-fatted by means of n-hexane, and the aglycones are extracted by means of ethyl ether, in the same manner as for Practical Embodiment 1, to yield 7.2 g of isoflavone aglycones. The yield of aglycones is improved by 60% by grinding at 50° C and by standing at that temperature for 1 hour.

Practical Embodiment 3

50 l of water at a temperature of 50° C is added to 10 kg of minimally denatured de-fatted soybeans (Nissei Soya Flour) and stirred for 1 hour. This is hot air dried by spray drying to

yield the refining raw material. The isoflavones are extracted by means of five times the volume of the refining raw material of hot 80% methanol and dried under reduced pressure to yield 103 g of a raw isoflavone fraction. This is redissolved in a small amount of methanol and passed through a ϕ 70 mm x 100 cm column packed with ODS-A Type 60-01 (manufactured by Yamamura Kagaku Kenkyujo KK) as the packing material and adsorbed.

Next, the phenolic acid and isoflavone glycosides are extracted by means of 40% methanol and discarded, and the residue is eluted by means of 80% methanol and dried under reduced pressure, to yield 9.5 g of aglycones.

Practical Embodiment 4

1 kg of *Diaion* HP-20 synthetic adsorbent (manufactured by Mitsubishi Chemical) is added to 25 l of soaking water that has been employed for soaking shelled soybeans after the manner of Practical Embodiment 1 and stirred for 1 hour to cause the adsorption of the isoflavone compounds. Next, the resin is filtered off and washed with 20% ethanol to remove the phenolic acid, and is eluted with 80% ethanol in order to yield the isoflavone compounds. After drying under reduced pressure, the mass of the isoflavone compounds is 1.1 g. Such isoflavone compounds contain approximately 40% of aglycones.

Applicant: Kikkoman Corp.

Reproductive Toxicology Review

REPRODUCTIVE AND GENERAL METABOLIC EFFECTS OF PHYTOESTROGENS IN MAMMALS

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Duke University Medical Center, Durham, North Carolina

Key Words: Phytoestrogens, Mammalian reproduction, Reproductive hormones, Gonadal steroids, Sexual differentiation, Hormone, General Metabolism, Ovarian function, Reproductive neuroendocrinology.

INTRODUCTION

Historically, phytoestrogens were first investigated when it was noted that ewes that grazed Australian clover pastures for prolonged periods of time became sterile. It was found that the active agents in the clover that precipitated sterility were estrogenic (1). Later a similar phenomenon was observed to occur in the California quail during dry years, when phytoestrogen concentrations in available forage were increased (2).

Phytoestrogens are defined as plant substances that are structurally and functionally similar to the gonadal steroid 17β-estradiol (E_2) or that produce estrogenic effects (3). There are three main groups of nonsteroidal dietary estrogens. Phytoestrogens include the isoflavones (i.e., genistein, genistin, daidzein, biochanin A, formononetin, and prunetin) and the coumestans (i.e., coumestrol and 4'-o-methylcoumestrol). Mycoestrogens of the resorcylic acid lactone group (i.e., zearalenone and zearalenol) are also commonly found (4). The structural similarity between these substances, endogenous mammalian estrogens (E_2 and estrone), and potent synthetic estrogens (diethylstilbestrol) have been studied (Figure 1). Isoflavones, the monocarboxylic derivatives of the 15-C flavones, and coumestans contain central structures of 15 carbons. Both of these groups are derivatives of 3-phenylchroman (Figure 1) and thus may be considered a single family of compounds (5). The fungal resorcylic acid lactones and endogenous estrogens possess central structures of 17 carbons.

The similarity among these compounds has led investigators to study the possibility that phytoestrogens might act on physiological processes and behavioral patterns to alter reproductive performance (3). If reproductive effects occur, then these compounds might have a role in the evolutionary success of herbivores, perhaps making the difference between survival and extinction for some species. It is possible that phytoestrogens, through mimicry of endogenous animal estrogens, function as defensive substances by which plants diminish the fertility of herbivores which feed on the plants (6). In effect, the phytoestrogens may be seen as one of the many variables determining animal fitness for survival. This argument is supported by noting that animal species differ in their sensitivity to phytoestrogens (7). Some species are relatively resistant to the estrogenic effects of these compounds, while others may suffer sterility as a result of prolonged ingestion of phytoestrogens. We have hypothesized that phytoestrogen-induced physiologic and behavioral effects in mammals are significant factors in the reproductive and therefore evolutionary success of the consuming species. We have initiated our analysis of this broad hypothesis by reviewing the available data relevant to the reproductive and general metabolic effects of phytoestrogens in mammals.

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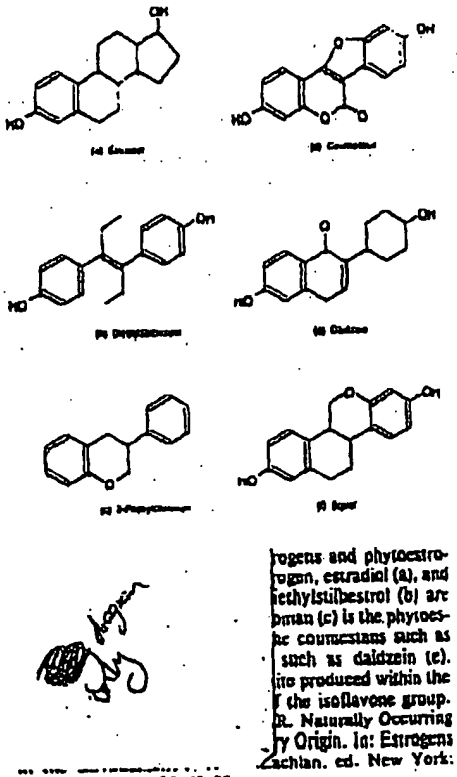

rogens and phytoestro-
rogen, estradiol (a), and
ethylstilbestrol (b) are
oman (c) is the phytoes-
he coumestans such as
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ite produced within the
f the isoflavone group.
R. Naturally Occurring
y Origin. In: Estrogens
achlan, ed. New York:
Elsevier Press, 1985; 69–85.)

Table 1. Some common plants that contain estrogenic substances

Alfalfa	Coffee	Oats	Rice
Anise	Date Palm	Orchard grass	Rye
Apple	Fennel	Palmetto grass	Sage
Barley	French Beans	Parsley	Sesame
Blue grass	Garlic	Peas	Soybean
Carrot	Green Beans	Pomegranate	Soya sprouts
Cherry	Hops	Potato	Wheat
Clovers	Liquorice	Rape	Yeast
	Marijuana	Red Beans	

PHYTOESTROGEN EXPOSURE

Sources of phytoestrogens

Phytoestrogens are produced by numerous Leguminosae and grasses, including many plants commonly consumed by man and livestock (Table 1). The estrogenic components are found in differing amounts in all parts of the plant, including the seeds, the flowers, the leaves, the roots, and the fruits. Concentrations in each tissue depend on plant type (4,8).

Of particular interest regarding possible human exposure is the presence of phytoestrogens in marijuana and coffee. It had long been suspected that the estrogenic effects of marijuana were due to Δ^9-tetrahydrocannabinol (THC), the major psychoactive compound. Smoking of marijuana significantly suppresses luteinizing hormone (LH) levels during the human menstrual cycle and shortens both the menstrual cycle and the luteal phase (9). Since these results agree with observations in ovariectomized rhesus monkeys injected intramuscularly (i.m.) with THC, it was assumed that the menstrual cycle effects of smoke inhalation would be exclusively due to the THC content of the smoke (10). However, crude marijuana extract and condensed marijuana smoke compete with estradiol for estrogen receptors in the uterus of rats, while in vitro studies detected no binding of cannabinoids to estrogen receptors (11). These findings show that marijuana contains estrogenic substances that may be affecting reproductive processes via cannabinoid-independent mechanisms. Furthermore, apigenin, a derivative of flavonoid phytoestrogens found in crude marijuana, is a moderately potent inhibitor of estradiol binding to uterine estrogen receptors (11). Differentiation between the suppressive effect of THC on LH and the estrogenic effects of marijuana *per se* remains unclear.

Another plant product which is commonly ingested for pleasure rather than nutrition is coffee. Like marijuana, coffee contains weakly estrogenic constituents, evidenced by the estrogenic effects of increased uterine-to-body weight ratio and total uterine protein content following administration of coffee extracts by gavage (12). Ultraviolet absorbance spectroscopy suggests that whatever this active compound may be, it does not belong to one of the three major classes of dietary estrogens (e.g., flavonoids, coumestans, or resorcylic acid lactones). Thus, coffee may contain an estrogen precursor that requires metabolic activation or a structurally unrelated estrogenic compound.

Metabolism, distribution, and clearance

The relative potency of a phytoestrogen depends upon the target tissue, functional state of the target tissue, the animal species involved, and the route and pattern of delivery. In addition, the fami-

ties of estrogenic compounds that occur in plants can be modified by metabolism within the herbivore or even by gut flora prior to uptake. Dietary isoflavone phytoestrogens undergo bacterial modification in the gastrointestinal tracts of animals to yield equol, a weak, nonsteroidal phytoestrogen (8,13,14). Following ingestion of estrogenic plants, a temporary 10- to 1000-fold increase in urinary equol takes place, while insignificant traces of the initially consumed phytoestrogens appear in the urine. Noteworthy is that the major urinary product following the consumption of genistein and biochanin A is p-ethyl phenol, and formononetin consumption yields both daidzein and equol as the major urinary products (4). Furthermore, gut microflora (14) convert daidzein to equol which in turn is absorbed and enters the enterohepatic circulation. Notably, it appears that not all people have the ability to convert other isoflavones to equol. This may be due to the absence of bacteria capable of the conversion of precursors to equol (as is the case in the sterile gut of newborns), the composition (subpopulations) of intestinal microflora present, the intestinal transit time, pH, or redox potential. These factors may be influenced by diet, host immunity, medication use, etc.

Receptor activity and interaction with endogenous estrogens

Phytoestrogens exhibit binding to endogenous estrogen receptors. Binding of phytoestrogens to estrogen receptors is supported by the finding that the larger the dose of phytoestrogen given an organism, the greater the displacement of bound tritiated (^{3}H) E_2 (15). It has also been reported that at very high dosages, all phytoestrogens exhibit more than 80% competitive binding to renal tumor cytosolic estrogen receptors (16). The structural requisites for estrogen receptor binding are met by phytoestrogens. For example, equol possesses a potency on the order of 10^{-3} the estrogenic activity of E_2 and contains a phenyl substituent also present in E_2 and in DES (Figure 1). The substituent considered to be a requirement for estrogenic activity is a hydroxyl group in the same position as the hydroxyl group in the benzene ring of E_1 (14). Another structural similarity which facilitates estrogen receptor binding activity of equol and other phytoestrogens is that the distance between C-3 and C-17 in E_2 is about equal to that between the two hydroxyls in equol.

Considering the large quantities of phytoestrogens ingested by many mammals including man, functionally significant estrogen receptor occupancy by phytoestrogens occurs. Since no phytoestrogen has receptor affinity equal to that of E_2 and the degree of DNA stimulation due to phytoestrogens appears to be substantially less than that evoked by E_2 (8), phytoestrogen actions could be either estrogenic or anti-estrogenic. In a relatively hypoestrogenic individual, receptor occupancy by weak (exogenous) estrogens would likely produce estrogenic effects, while in a normally estrogenized individual, large amounts of weak estrogens might diminish the effective estrogenic activity by competition with E_2.

REPRODUCTIVE EFFECTS IN MAMMALS

Phytoestrogens have been shown to influence virtually every aspect of the mammalian reproductive process via effects on the morphology and physiology of reproductive organs and alteration of sexual behavior. The changes may be reversible or irreversible, depending on the duration and dose of exposure to the phytoestrogens.

Cervix

A pubertal pattern of cell differentiation has been noted in ewes rendered sterile by chronic ingestion of phytoestrogens (17). Among these changes, the cervix assumes a uterine pattern. Folds present in the cervix fuse, resulting in loss of cervical crypts, and the cells of the lamina propria become like those of the uterine stroma. Furthermore, glands having histochemical reactions reminiscent of uterine glands become plentiful in the cervix. Such an increase in abnormal glands may be responsible for the different composition which the cervical mucus takes in sheep with "clover disease." At low phytoestrogen dosage, the cervical mucus has a lower viscosity, not due to a higher water content, but rather due to a decreased concentration of glycoprotein — the component of mucus that affords its consistency. The level of glycoprotein seems to respond to the duration of exposure to the phytoestrogen rather to the dosage of the agent. This change in the cervical mucus compounds the anatomical compromise of the cervix such that the cervical reservoir for sperm in the ewes is greatly reduced. Since sperm recovered from the cervices of clover-affected ewes exhibit decreased motility (17), it appears that the phytoestrogen effect makes the mucus relatively "hostile" in the classic sense of cervical factor infertility. Such spermatoxicity is not understood in general nor in this specific case.

At higher phytoestrogen dosage, both higher volume and water content of cervical mucus are

observed in ewes (17,18), thus indicating that both cervical glycoprotein production and water excretion in the mucus are affected.

The cervical effects of phytoestrogens likely depend upon estrogen receptor mediation. In ewes, phytoestrogen treatment increases the rate of protein and glycoprotein synthesis and the number of estrogen binding sites in the cervix, but binding affinity remains unchanged (19). This finding implies that exogenous estrogen not only occupies the available binding sites, but stimulates the local production of more sites. Such receptor "up-regulation" may make the tissue more sensitive to estrogen action, and, if estrogen exposure continues, the cervical alterations would become more exaggerated.

Uterus

Pronounced uterine effects of phytoestrogens are also observed. The most notable uterine change that occurs is a marked increase in its weight relative to body weight, which constitutes the classic bioassay for estrogen action. A dose-dependent uterine weight increase is precipitated by acute administration of an extract of the Indian herb *Achyranthes aspera* in rats and hamsters at contraceptive dosage (75 mg/kg) and with as little as 1/20 this dosage (20). Similar results have been observed in mice, rats, and hamsters with only 1/40 contraceptive dose of ferujol extract (21). Stob (4) suggests that this hypertrophy of the uterus is the result of "typical estrogenic mechanisms," implying estrogen-receptor mediation. However, a more complex response to daily s.c. injection of female lambs with the phytoestrogen β-sitosterol has been reported, in which uterine weight increases for the first two weeks of treatment but markedly decreases over the next six-week period (22). Plausible explanations for such biphasic results include receptor "down regulation" and induction of metabolic enzymes with enhanced clearance of β-sitosterol. Similar results were obtained using ovariectomized ewes as the model (23).

Another manifestation of the uterotropic effect of phytoestrogens is seen in ewes suffering from infertility due to prolonged exposure to these agents. A marked increase in activity of some uterine enzymes and uterine DNA, protein, and glycoprotein synthesis occurs in such sheep (19). This observation indicates that at least a portion of the uterine weight gain is true hypertrophy rather than simply edema. At the same time, lower levels of lipids within the uteri of sheep fed phytoestrogen suggest inhibition of synthesis or increased utilization of lipids within this organ (22). Thus phytoestrogens may be affecting different enzymes in different fashions, stimulating the activity of some while blocking the action of others. It is noteworthy that the uterine RNA-to-DNA ratio decrease that occurs following ovariectomy is smaller in clover-affected than in normal ewes. This response is accompanied by less regression of the uterus in clover affected ewes than in controls. These findings indicate that phytoestrogenic action may be mediated via differentiations similar to those induced by hormonal steroids during fetal development (24).

Gross structural lesions of the uterus may also result from phytoestrogen exposure and could account for some instances of permanent sterility. Lesser lesions entail the proliferation of cystic endometrium, myometrial fibrosis, and endometrial fibrosis (13). These lesions could certainly compromise normal implantation of the conceptus. The most severe structural failure, complete uterine prolapse, is known to occur in some species following ingestion of some dietary estrogens (mycoestrogens) and obviously disrupts the reproductive process.

It is not clear whether phytoestrogens play any role in pregnancy wastage, but some plant preparations have been used as abortifacients. *Achyranthes aspera*, a common Indian herb claimed to possess abortifacient activity, did induce abortion in mice and rabbits, but failed to show similar effects in rats (20). It is uncertain whether a phytoestrogen is the active agent of *Achyranthes* that brings about abortion, but support for that possibility derives from the finding that miroestrol, a phytoestrogen from a legume tree root, is used by Burmese and Thai women in plant extract form to induce abortion (25). The mechanism for such an abortifacient action of these compounds is unstudied and any effects of phytoestrogens on uterine contractility *per se* have not been determined in either the gravid or nongravid state.

Phytoestrogen effects on uterine function may relate to alterations in activity of several enzymes. Under normal circumstances, oxidative enzymes in the uterus show slight reactions in the endometrium and uterine glands, but after administration of β-sitosterol, these weak reactions are curtailed (22). Such an inhibition of oxidative enzymatic activity in the uterine endometrium and glands may reduce local energy production due to an inability to replenish NAD+ and NADP+. This circumstance would diminish the ability of the uterus to contract and might decrease secretory capabilities of the uterine glands.

Alkaline phosphatase in the uterine tissue of ewes also responds to β-sitosterol in a biphasic pat-

tern. Alkaline phosphatase activity increases over the first two weeks of daily β-sitosterol injections and decreases over the second two weeks of injections (22). This disturbance in alkaline phosphatase activity may alter cell permeability and transport of nutrients by uterine cells.

Acid phosphatase activity in the uterus decreases with increasing dose and time of daily β-sitosterol treatments over an eight-week span (22). Such an inhibition would decrease free phosphorous, and may relate to the more general observation of decreased plasma phosphorus levels in exposed animals.

Uterine cholinesterase activity also decreases following β-sitosterol treatment, as evidenced by its diminished activity towards acetylthiocholine (22). This inhibition of activity is accompanied by a downward shift in sodium ion transport and decreased sodium in the uterine luminal fluid. It is not clear whether effects on sodium transport and cholinesterase activity are coincidental or truly associated processes in this instance.

Ovaries

While many anatomical effects of phytoestrogens have been described, physiologic changes in the reproductive tract are more subtle, but perhaps more consequential. Ovarian cyclicity may be disrupted by phytoestrogen exposure in mammals and birds (2,14,25,26), but interruption of ovulation due to short-term phytoestrogen ingestion is reversible (26). It is plausible that human vegetarians may have ovulatory dysfunction but suffer no other obvious physiologic abnormalities due to their diets (14). Abnormalities of ovulation may be due to direct ovarian actions since administration of β-sitosterol to ewes inhibited follicular development and altered the size distribution of follicles (22). Follicles were observed to show degeneration with intrafollicular hemorrhage and the development of shrivelled oocytes with lipid inclusions. The suggestion of a direct ovarian action of phytoestrogens in perturbing follicular maturation may be supported to some extent by a study which showed that in rats intraperitoneal administration of an extract from a plant species known to contain high concentrations of phytoestrogens inhibited follicular maturation (26). Obviously, these studies cannot distinguish between direct ovarian and indirect effects on follicular growth.

More direct evidence that the follicle may be a site of phytoestrogen activity derives from in vitro cultures of bovine granulosa cells. In this system, lower dosages of genistein and biochanin A increased progesterone synthesis while higher dosages inhibited progesterone synthesis (27). Since progesterone is essential in the establishment and maintenance of pregnancy, such an inhibition of progesterone production would be a plausible explanation for both failure of conception and early pregnancy wastage.

The possibility that phytoestrogens might be toxic to oocytes or early embryos was suggested in a single study (7). Mice fed coumestrol and then mated produced degenerate embryos exhibiting unevenly distributed cytoplasm and lack of symmetry in size among blastomeres, suggesting alterations in cleavage rates. Extensive vacuolization found in the ova also suggests that failure of fertilization of these ova may account for part of the observed decrease in litter size in mice fed coumestrol.

The activities of two ovarian enzymes appear to be influenced by phytoestrogens. First, low doses of phytoestrogen inhibit 17,20-lyase in bovine granulosa cells (27). This effect could profoundly alter the pattern and capacity of the steroidogenic pathways within the follicle or corpus luteum. The precise mechanism by which this effect occurs is unproven. Second, alkaline phosphatase in the ovaries is affected by phytoestrogen exposure (22). While the overall alkaline phosphatase activity is about equal in the ovaries of β-sitosterol-treated and control ewes, the control ewes show an intense reaction in the zona pellucida with a weak reaction in the interstitial tissue. Treated ewes exhibit an opposite response. Thus, a reversal of activities is seen where phytoestrogen is acting both to stimulate and to inhibit the same enzyme in two different sites within the ovary. While a mechanism for this action is not known, such changes in the activities of ovarian enzymes might compromise ovulation and increase the incidence of follicular degeneration in animals treated with phytoestrogens.

CNS/pituitary

Some phytoestrogen effects on ovarian function appear to result from indirect action on the secretion of gonadotropic hormones (7). In this context, there are four possible mechanisms of phytoestrogen action: 1) they are E_2 agonists, 2) they are E_2 antagonists, 3) they act as both E_2 agonists and antagonists, and 4) they act in a nonestrogenic capacity. Available information best supports the third of these possibilities (mixed agonist–antagonist effects). The site of phytoestrogen action could be the CNS (especially hypothalamus), the pituitary, or the gonad (see previous section).

The effect of intraperitoneal injection of phytoestrogen-rich *Dieffenbachia amoena* extract in rats on LH, follicle-stimulating hormone (FSH), prolactin (PRL), progesterone, and E_2 have been studied (26). In treated rats, levels of LH, FSH, and progesterone increased for doses of 2.5, 5.0, and 10.0 mg/kg of extract, while the levels of PRL and E_2 decreased at the same dosages. Progesterone levels showed a biphasic response, increasing at low doses of the extract (26), but decreasing at higher doses (27). Since no obvious single mechanism would explain all of these pituitary and ovarian hormonal changes, the extract may contain more than one endocrinologically active substance, or more than one site or mechanism of action might be involved.

There are data to suggest that phytoestrogens act both at CNS and pituitary levels to alter gonadotropin secretion. In both ovariectomized ewes (23) and intact clover-affected ewes (17), the best explanation for the impairment of gonadotropin secretion was a hypothalamic/CNS action. In particular, in clover-affected ewes, an LH surge could not be elicited by exogenous E_2 administration (consistent with loss of positive feedback), but the LH secretory response to exogenous gonadotropin-releasing hormone was normal (17), suggesting no pituitary effect. Our own data (28) show that acute phytoestrogen administration can alter GnRH-induced LH secretion in ovariectomized rats and thus suggest that the pituitary may be a site of phytoestrogen action in other situations.

Interactions between reproductive effects of phytoestrogen exposure and photoperiod in seasonal breeders have been investigated. In normal intact ewes, the frequency of LH pulses and plasma LH concentration are higher during breeding season than during anestrus season. In clover-diseased ewes, the frequency of LH pulses and LH concentration during breeding season are nearly the same as in normal ewes. In contrast during anestrus season, these LH pulse parameters remain at the high level of breeding season in clover-affected ewes, rather than decreasing as in normal ewes (18). These results suggest that a dissociation of normal photoperiod controls from the LH pulse generator may result from prolonged phytoestrogen exposure.

In ovariectomized ewes given estradiol implants, LH pulse frequency and amplitude vary seasonally, rather like the pattern seen in intact ewes. This seasonal variation in LH pulse frequency in ovariectomized ewes could depend upon extraovarian steroids from the adrenal glands, other intrinsic photoperiod-dependent CNS functional changes, or dietary estrogens. Results from one study suggest that dietary coumestrol decreases the amplitude of LH pulses but fails to affect the frequency of LH pulses or FSH concentrations during the breeding season (23). During anestrus, coumestrol does not alter any of these variables. Thus, coumestrol could only be partially responsible for the seasonal decrease in LH pulse frequency in ewes.

Sexual behavior

Changes in sexual behavior due to phytoestrogen exposure parallel the known physiologic effects. Clover-diseased ewes are slower than normal ewes to exhibit estrus behavior in response to either a single or several daily doses of E_2 (17,29,30). Accompanying the delayed estrus is a retarding of the first mount of the ewes by the ram, although the number of days on which the ewes allowed the ram to mount them does not significantly differ from controls. A delay of estrus in mice fed coumestrol also occurs (7), implying an antiestrogenic effect.

Apparent defeminization of the sexual behavior response following consumption of phytoestrogens is displayed by clover-affected ewes. These ewes show aggressive behavior, such as challenging and head hunting of rams and other ewes, sooner than control ewes following administration of testosterone (17). At the same time the ewes are slower in showing female behavior, such as standing to be mounted by a ram. Furthermore, clover-affected ewes exhibited less soliciting behavior than normals. However, the number of ewes that stood to be mounted decreased equally over the five-week period during which daily testosterone injections were given (30). Relative to controls, clover-diseased ewes exhibit a significantly greater degree of courting behavior 28 but not 21 days following treatment with testosterone. Other courting behaviors that are less hormonally dependent, such as anal and genital sniffing by the ewes, are not altered (17,30). While mechanisms for these behavioral effects are not known, we do know that females and males have similar numbers of estrogen binding sites in the hypothalamus, but estrogen-receptor complexes appear to have shorter nuclear acceptor occupancy in males than in females (31). Behavioral changes in clover-affected ewes could result from a change as simple as a decrease in nuclear acceptor occupancy by estrogen-receptor complexes.

E_2 causes a dose-dependent increase in the incidence and duration of hormone-dependent behaviors in ewes (Table 2), whereas E_2 has no effect on hormone-independent behaviors (30). The E_2

Table 2. Estradiol-dependent and -independent behaviors in ewes

Hormone-dependent behaviors	Hormone-independent behaviors
Active soliciting	Squatting
Standing for mounting	Looking over shoulder
Allowing ram to mount	Tail fanning
	Kicking

induced behaviors occur less in phytoestrogen-affected ewes than in normals, while E_2 independent behaviors occur with equal frequency in control and clover-diseased ewes. Since general behavior appears normal but female sex-specific behavior is compromised in phytoestrogen-treated ewes, reproductive success could be compromised on a behavioral basis. The relationship of phytoestrogen-induced anatomic changes in the external genitalia and sexual behavior is not defined, but coital biomechanics could be altered as a result of such end-organ effects. While vulvar and vaginal hypertrophy has been noted in various animals, masculinization has been observed in ewes (17) with clitoromegaly and fusion of the ventral commissure. Upon removal from estrogenic pasture, these changes do not reverse and could, therefore, permanently alter sexual function.

Phytoestrogenic effects in males appear to be consistent with expectations for exogenous administration of bioactive estrogen. Coumestrol increases teat length in wethers (23) and stimulates mammary hypertrophy in intact males. Rams grazed on estrogenic clover have reduced sperm counts (14), but it is not clear whether fertility is affected.

GENERAL METABOLIC EFFECTS IN MAMMALS

Protein synthesis

Some data suggest that phytoestrogens affect levels of plasma proteins. The effects of β-sitosterol on plasma concentrations of albumin, alpha-globulin, beta-globulin, gamma-globulin, and fibrinogen have been studied (32). Normal functions of these proteins are indicated in Table 3 (33). Even though total plasma protein concentration in mice is unaffected by s.c. administration of β-sitosterol, daily 25 to 100 μg injections of the agent increase four of the plasma proteins, but significantly decrease the gamma-globulin complex. The mechanisms of action of phytoestrogens in this system

Table 3. Plasma protein fractions affected by β-sitosterol*

Protein	Function	Effect of β-Sitosterol
Serum albumin	Regulation of blood volume; transport of fatty acids	Increase
Alpha-globulins	Transport of lipids, thyroxine, adrenal cortical hormones, and copper	Increase
Beta-globulins	Transport of lipids, iron, and hemes	Increase
Gamma-globulins	Act as most of the circulating antibodies	Decrease
Fibrinogen	Precursor to fibrin of blood clots	Increase

*(See reference 32).

are not established. It is likely that the phytoestrogens stimulate hepatic protein synthesis but inhibit production of gamma-globulins by lymphoid tissues. It is possible that the increased alpha-globulin concentration is a compensatory occurrence to erythrocyte count reduction that occurs following administration of β-sitosterol, thereby maintaining normal blood viscosity in the absence of normal erythrocyte concentration. The increase in the beta-globulin–fibrinogen complex appears to be correlated with its affinity for binding phosphorus. This affinity increases in response to β-sitosterol (32).

Enzyme activity of the liver

Phytoestrogens influence enzymes in nonreproductive as well as reproductive tissues. A relation between diet and synthesis of three enzymes in the liver of cheetahs has been shown. The affected enzymes, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase, decrease in amount when cheetahs are taken off a diet high in soya bean content (thus high in phytoestrogen content) and given a chicken diet (13).

Inorganic plasma constituents

Phytoestrogens induce mineral changes in the blood. Subcutaneous injections of 25, 50, 75, or 100 μg of β-sitosterol increase calcium levels in mice, while doses of 5 or 10 μg of the phytoestrogen have no effect on calcium levels (34). Since E_2 inhibits bone mobilization, β-sitosterol may act by causing a decrease in E_2 levels via inhibition of gonadotropin secretion from the pituitary. Decreased ovarian E_2

production might then result in increased bone mobilization and increased serum calcium. Surprisingly, blood plasma phosphorus levels decrease following administration of 5 to 75 μg doses of β-sitosterol in mice, but show little change in response to a 100 μg dose (34). Decreases in phosphorus could be due to an enhanced rate of storage in an extravascular compartment, increased utilization of phosphorus by tissues, or increased renal clearance.

While β-sitosterol doses of less than 5 μg fail to change plasma magnesium levels, higher doses decrease plasma magnesium and increase both hepatic and intramuscular magnesium (34). Since magnesium is a smooth muscle relaxant, changes in uterine or tubal smooth muscle motility could result indirectly from this phytoestrogen action.

PHYTOESTROGENS IN HUMAN DISEASE

Deleterious roles

Phytoestrogens have been suggested to play both deleterious and beneficial roles with regard to illness. In the diets of cheetahs, phytoestrogens cause vascular hepatic lesions, in which the centrilobular and sublobular hepatic veins are partially or totally occluded (13). The possibility of human hepatic dysfunction must therefore at least be considered.

Vascular disease may be correlated with the consumption of dietary phytoestrogens (35). Coronary heart disease has been suggested to be associated with phytoestrogens consumed indirectly through the milk of cows; that is, the lactating cow consumes the phytoestrogens while grazing and, in turn, phytoestrogens in cow's milk are consumed by humans. One basis for this proposal is that phytoestrogens have more structural similarity to DES, a potent synthetic estrogen found to have atherogenic properties, than to endogenous estrogens such as E_2. The higher rate of coronary heart disease in human males might be explicable in part if human females are found to be better able to metabolize and excrete phytoestrogens.

Dietary estrogens could be a factor in cancer initiation in hormone responsive tissues, but no such instances have been demonstrated. Certainly phytoestrogens bind to both rat and human mammary tumor tissue and show competitive binding for mammary tissue E_2 receptors (15) raising the possibility of stimulation of estrogen-dependent neoplasms.

Beneficial roles

Estrogens have two opposing effects on cancer, depending on dosage. Large doses inhibit breast cancer tumor development and suppress growth of tumors already present, but small doses seem to promote tumor development and stimulate growth (36). This duality extends to phytoestrogens. Phytoestrogens may stimulate or inhibit tumor growth (8,14). One mechanism by which phytoestrogens may manifest their antitumor effects is blockade of estrogen receptors and uncoupling of receptor-mediated response. Thus the ability of endogenous estrogens to support tumor growth would be reduced. Indirect demographic support for a phytoestrogen-mediated reduction in cancers of hormone-responsive tissues might derive from the observation that women in countries consuming vegetarian diets have a lower incidence of breast cancer than in societies where a meat and vegetable diet is consumed (37).

Phytoestrogens may have antiviral and fungicidal properties (37), but a mechanism is not known. Support for the notion that this group of compounds could have such properties may lie in noting that the antifungal drug, ketaconazole, is also a potent inhibitor of some steroidal enzymes.

Plant estrogens have been implicated in the reduction of serum cholesterol levels in humans and animals with hypercholesterolemia. Such action is likely related to the role estrogens play in the metabolism and interaction of lipoproteins with regulation of cholesterol (8).

A final beneficial phytoestrogenic effect is alleviation of vasomotor symptoms in menopausal women. Historically the Chinese have used herbal medicine to treat "hot flushes." These herbal medications work as well as Premarin (an equine conjugated estrogen) in the mitigation of these symptoms in women with natural menopause (38). Similarly, the mycoestrogen, zearalanol, has been reported to reduce the incidence of hot flushes in women with surgical menopause (4). These effects would be consistent with the expected estrogenic properties of these compounds.

CONCLUSION

Phytoestrogens influence mammalian reproductive processes and can thereby compromise the reproductive success of individual mammals and possibly function as a selective environmental factor for populations. While phytoestrogens have a few propitious effects, the majority of the effects are noxious. These compounds act through their similarity to endogenous estrogens and compete with the endogenous estrogens for binding sites.

Short-term effects of phytoestrogens seem to result from their mixed agonist–antagonist effects on estrogen-mediated processes in mammals. Since long-term exposures can produce persistent, even permanent anatomic, physiologic, or behavioral changes, phytoestrogens must affect the differentiation of some reproductive tissues and irreversibly alter the integration of mammalian reproductive processes in susceptable species.

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COMMENTARY

The Role of Soy Products in Reducing Risk of Cancer[1]

Mark Messina, Stephen Barnes*

Since the initial recognition that diet plays a role in the etiology of certain cancers, particularly cancers of the breast and colon, considerable progress has been made in identifying dietary patterns associated with cancer risk. There is general agreement that a high-fat, low-fiber diet, like that consumed by much of the industrialized world, increases cancer risk and that plant-based diets, rich in whole grains, legumes, and fruits and vegetables, are protective. It has been, however, considerably more difficult to identify specific foods, types of food, or components of foods that influence cancer risk.

The recent workshop on The Role of Soy Products in Cancer Prevention, sponsored by the National Cancer Institute, had two objectives: 1) to evaluate the role of soybeans, food products derived from soybeans, and specific components of soybeans in the dietary prevention of cancer and 2) to recommend research initiatives and approaches for further studies of the effect of soy intake on human cancer risk. The meeting was chaired by Stephen Barnes and organized by Mark Messina.

Isoflavones in Cancer Prevention

Kenneth Setchell, Donna Baird, and Barnes discussed the potential role of isoflavones in the prevention of cancer. Setchell reviewed the history of phytoestrogens (*1*), noting that plants were first observed to induce estrus in animals in 1926. Over 300 plants are now known to possess estrogenic activity (*2,3*). In 1946, the infertility observed in Australian sheep that grazed on a certain type of subterranean clover was attributed to the high isoflavone content of this plant (*4*). Ruminal bacteria in these animals convert plant isoflavones into the mammalian isoflavone equol, which, following absorption, may suppress the pituitary gonadotropic axis. Equol, a weak estrogen possessing about 0.2% of the biological activity of estradiol, was first identified in human urine in 1982 by Setchell et al (*5,6*). Setchell's further interest in the potent estrogenic effects of soybean isoflavones was stimulated coincidentally. He discovered that the soy component of diets fed to captive cheetahs, which was added for economic reasons, was responsible for the severe breeding problems in these animals (*6,7*).

Setchell noted that isoflavone metabolism has been studied in humans, although only superficially. In one study, subjects fed 40 g of soy daily were found to have urinary levels of equol as much as 1000-fold higher than baseline values (*8,9*). The low levels of urinary equol in two of the six subjects in this study indicate that the intestinal microflora (*10*) participate in isoflavone metabolism and that isoflavones undergo enterohepatic circulation (*10*). Improved analytical methods (*11,12*) have led to the realization that equol represents only a small fraction of the total amount of isoflavone in urine and that conjugates of the soybean isoflavones daidzein and genistein are the major forms present. The high levels of isoflavone in urine in subjects fed soy suggest that these compounds are likely to elicit a biological response (*13*).

Setchell concluded his presentation with a reminder (*a*) that all weak estrogens can also have antiestrogenic activity; (*b*) that tamoxifen, which has been used therapeutically for breast cancer, is structurally related to some of the phytoestrogens; and (*c*) that vegetarians, who may have a lower risk of certain cancers, excrete higher levels of phytoestrogens. These findings have led to collaborative studies by Barnes, Setchell, and associates (*14*), who used an animal model designed to test the hypothesis that phytoestrogens have a role in reduction of breast cancer risk.

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[1]Report of a workshop held June 26-27, 1990, at the Guest Quarters Hotel in Bethesda, Md. Workshop members were Donna Baird, National Institute of Environmental Health Sciences, Research Triangle Park, NC; Stephen Barnes, University of Alabama at Birmingham, Birmingham, Ala; David L. Brandon, Western Regional Research Center, United States Department of Agriculture, Albany, Calif; James A. Duke, Agricultural Research Service, United States Department of Agriculture, Beltsville, Md; Ernst Graf, The Pillsbury Co, Minneapolis, Minn; Ann R. Kennedy, University of Pennsylvania Medical School, Philadelphia; Renee M. Kozalak, Iowa State University, Ames; Irvin E. Liener, University of Minnesota, St. Paul; Mark Messina, National Cancer Institute, Bethesda, Md; Frank L. Meyskens, University of California, Irvine, Calif; A. Venket Rao, University of Toronto, Ontario, Canada; Kenneth D. R. Setchell, Children's Hospital, Cincinnati, Ohio; Bernie P. Szuhaj, Central Soya, Fort Wayne, Ind.

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mes began by observing that Oriental women, who have incidence rates of breast cancer (*15*), consume larger iounts of soy products than do most American women. How-er, although fertility and reproduction in animals are adverse-affected by ingestion of plant isoflavones, the amount of ıflavones in soy products consumed by Oriental women does t appear to affect their reproductive capacity.

Barnes discussed the recent animal study conducted in col-ɔoration with Setchell and other investigators (*14*). In that udy, consumption of soybeans significantly decreased chemi-ılly induced rodent mammary cancer. Rats were fed one of ıur soy products: powdered soybean chips consisting of un-urified soybeans, both raw and autoclaved; soy protein isolate ɔmposed of 91% protein; soy molasses, a concentrate of the queous alcohol extract of soy flour; and aqueous alcohol-ex-acted soy protein concentrate. All diets were isocaloric and ıonitrogenous and produced similar weight gain among the nimal groups throughout the study.

The first three products, all of which are rich in isoflavones, nhibited mammary tumorigenesis induced by 7,12-dimethyl-[a]anthracene or methylnitrosourea, while the aqueous al-ol-extracted soy protein concentrate, which had a low ontent of isoflavones, did not. Whether the soybeans were raw ɔr cooked made no difference in the degree of inhibition of nammary cancer; cooked soybeans were shown to be devoid of ɔrotease inhibitor activity.

Barnes said the reduction in levels of mammary tumor estrogen receptors induced by the powdered soybean chips paralleled the inhibition of tumorigenesis and supported the hypothesis that the isoflavones exerted an antiestrogenic effect. Interestingly, however, this was not the case for the soy protein isolate. The decrease in levels of mammary tumor estrogen receptors was smaller than predicted from the degree of tumor inhibition, he said, suggesting that the antiestrogenic effect of isoflavones may not be the primary mechanism responsible for inhibition of tumorigenesis. Therefore, Barnes concluded, the anticarcinogenic activity of isoflavones may not be limited to tumors containing a functional steroid receptor system. Alterna-ɔi mechanisms of action may include inhibition of the activity rosine protein kinases (eg, epidermal growth factor receptor ɔsine kinase) (*16*), DNA topoisomerase II (*17*), and ribosomal S6 kinase (*18*), as well as induction of specific cytochrome P450s (*19*).

Baird, before describing her recent study of the effects of feeding soy to postmenopausal women (manuscript in preparation), cited the concern of the National Institute of Environmental Health Sciences about the possible effects of low-level environmental estrogens on health. In her study, changes in estrogenic activity in postmenopausal women consuming soy over a 4-week period were examined. Soy was chosen for this study because of its high estrogenic activity (*20,21*), its increasing use in the United States, and the variety of products derived from soy and because soy consumption would not adversely affect nutritional status (*22*). Subjects consumed daily one main soy dish (½ cup of soybeans or 38 g of texturized vegetable protein) and two soy snacks—either soy chips (a roasted soybean product) or a spread for crackers made from the whole soybean. The estimated isoflavone content was about 200 mg/day, the equivalent of about 0.3 mg/day of conjugated steroidal estrogen, assuming that the estrogenic activity of phytoestrogens is about 0.1% that of conjugated estrogen.

Baird said preliminary findings indicate that, compared with control subjects, significantly more women fed soy exhibited an estrogenic response, as demonstrated by an increase in the number of superficial cells of the vaginal epithelium. She remarked that postmenopausal women were chosen for this study because of the decision to examine the estrogenic rather than the antiestrogenic effects of plant phytoestrogens. In premenopausal women with relatively high estrogen levels, the antiestrogenic effects of soybeans may have been observed.

Protease Inhibitors

Ann Kennedy, David Brandon, and Irvin Liener focused their attention on the soybean protease inhibitors. Kennedy reviewed her work, as well as that of others, in the field of protease inhibitors and cancer prevention. She noted that the soybean-derived Bowman-Birk protease inhibitor (BBI) either inhibits or prevents development of experimentally induced colon (*23*), oral (*24*), lung (*25*), liver (*23*), and esophageal cancers (von Hofe E, Newberne P, Kennedy A: unpublished observations). Protease inhibitors, at the levels used in these studies, do not adversely affect animal growth. Kennedy noted that the anticarcinogenic effect of the BBI is thought to stem from its ability to inhibit chymotrypsin activity (*26*), but results also suggest an important role for trypsin inhibition in suppression of the promotional stage of carcinogenesis (*27*). She said in vitro work indicates that protease inhibitors prevent conversion of normal cells to the malignant state even at very late stages in carcinogenesis but that they have no effect on cancerous cells (*28*). Protease inhibitors are unique in that they cause an irreversible suppressive effect on the carcinogenic process. They have also been shown to suppress oncogene expression and to inhibit carcinogen-induced protease activity (*29*).

Kennedy said recent data suggest that the antigrowth effects of raw soybeans commonly attributed to protease inhibitors may actually be due to an unidentified factor(s) (*30*). Furthermore, in human populations consuming soybeans, the connection between pancreatic enlargement and protease inhibitors observed in animals has not been seen. In fact, incidence of pancreatic cancer is decreased in these groups (*31*). Kennedy noted that in vitro comparisons of the pure BBI with an extract of soybeans containing BBI indicate that the activity of the soybean extract could be directly attributable to BBI (*26*). However, she said an in vivo study suggests that the extract may contain an additional anticarcinogenic agent working in conjunction with the BBI (*26*). The extract contains approximately 50% protease inhibitor; the remaining content is unknown, but it may include isoflavones as well as other potential anticarcinogens. Kennedy commented that the lowest effective dietary levels of protease inhibitors used in these animal studies (0.1%) could be achieved by humans by modifying the diet to include soy products.

Brandon discussed the measurement of protease inhibitors in soybeans and soy products, noting the concern of the Agricultural Research Service of the United States Department of Agriculture (USDA) over the possible adverse effects of

protease inhibitor intake in humans, particularly in infants (*32*). Enzyme-linked immunosorbent assays (ELISA), using monoclonal antibodies, have been developed for the measurement of two different protease inhibitors found in soybeans—BBI and Kunitz trypsin inhibitor (KTI) (*33,34*). These procedures are suitable for quantifying residual protease inhibitor levels in foods. A variety of processed soy products, a series of soybean flours derived from seeds in the USDA Soybean Germplasm Collection, and the soybean isoline L81-4590 (lacking KTI) (*35*) have been analyzed. Brandon noted that an important observation from the ELISA analysis of heat-treated soy flours derived from the isoline was that KTI, not BBI, is responsible for the heat-stable activity of commercial soy flour that inhibits trypsin activity (*36,37*). The microenvironment of the soy flour appears to promote heat inactivation of BBI to a greater extent than it affects KTI. This finding contrasts with the results of work showing that BBI is relatively heat stable in the pure form (*38*). Moisture, fat content, the presence of agents that influence changes in disulfide bonds, and interactions with other constituents, such as carbohydrates, appear to influence the denaturation of inhibitors (*39*).

Brandon said analysis of infant formula has revealed that active KTI and BBI, when measured on the basis of weight per gram of protein, are reduced to about 0.1% of their levels in raw soy (*40*). An infant on a diet consisting exclusively of soy formula would consume about 10 mg of active KTI plus BBI per day. In toasted (autoclaved) soy flour, 20%-30% of the KTI activity remains, while all of the BBI is inactivated. Analysis of tofu (soybean curd) has revealed that the protease inhibitor content varied significantly among the samples, from 4 to 30 μg of BBI and from 5 to 16 μg of KTI per gram of product. The protease inhibitor content of several soy protein isolates also varied, as much as 20-fold. Not unexpectedly, there was also a wide variation in the protease inhibitor content among varieties of soybeans. Brandon suggested that food-processing strategies could be combined with genetic approaches to optimize the protease inhibitor content of soy products.

Liener reviewed research on the potential adverse effects of consuming protease inhibitors, first noting that most work has been done with small experimental animals (*41*). Consumption of raw soybeans has two major effects: growth inhibition and pancreatic enlargement. Rats consuming raw soy flour for extended periods develop adenomatous nodules involving acinar cells of the pancreas (*42*). Additionally, raw soy flour consumption potentiates the effect of pancreatic carcinogens (*43*). In a study by Liener et al (*44*), heat treatment of raw soybeans almost completely eliminated this potentiation, while the addition of protease inhibitors to the heated product restored most of the pancreatic enlargement observed with raw soy, suggesting that protease inhibitors are at least partly responsible for pancreatic enlargement.

Liener noted that the varied response to raw soy flour among species is particularly important. Rats, mice, chickens, hamsters, and young, growing guinea pigs all exhibit pancreatic enlargement in response to protease inhibitors, while dogs, pigs, calves, and monkeys do not (*45*). Growth inhibition induced by soybean products is thought to result from a deficiency of the sulfur-containing amino acids caused by the dramatic increases in fecal levels of endogenous protease enzymes, particularly trypsin and chymotrypsin, two enzymes that are rich in these amino acids (*46*).

Commenting that pancreatic enlargement apparently stems from elevated serum levels of the hormone cholecystokinin, Liener commented that pancreatic enzyme secretion is inversely related to the level of trypsin in the intestine, a process regulated by cholecystokinin. This hormone stimulates the pancreas to produce trypsinogen, but because the protease inhibitors combine with trypsin, the suppressive effect of trypsin on intestinal release of cholecystokinin is eliminated (*47*).

Liener raised the question: Can the effects of protease inhibitors in small animals be extrapolated to humans? A negative feedback system in humans has been observed (*48*). Directly supplying BBI or raw soy flour to the duodenum causes an increase in secretion of pancreatic enzymes (*48*) and in blood levels of cholecystokinin (*49*). (BBI, in contrast to KTI, survives in gastric juice.) Despite these observations, he said, it is not possible at this time to accurately assess the health consequences of consuming processed soy products.

Phytosterols and Saponins

A. Venket Rao presented evidence for reduction of colon cancer risk by phytosterols and saponins. Both substances are common constituents of plants, but the concentration in soybeans is particularly high. Phytosterols are structurally similar to the animal sterol cholesterol. They inhibit cholesterol absorption and are almost quantitatively recoverable in fecal material, indicating that very little intestinal absorption occurs (*50*). Soybeans are a major contributor of phytosterols to the diet, particularly β-sitosterol (90 mg/100 g edible portion of the soybean) (*51*). Soybean oil is potentially an important source of phytosterols, but upon refinement and hydrogenation, phytosterol levels are reduced from 315 mg to 217 mg and 132 mg, respectively, per 100 g of oil (*51*). Dietary phytosterol intake among populations differs dramatically: the typical western diet contains about 80 mg/day, while Japanese and vegetarian diets provide about 400 and 345 mg/day, respectively (*52,53*).

In addition to the phytosterols, whole soybeans contain significant amounts of saponins, about 5% of dry weight (*54*), while tofu contains approximately half that much. Saponins are amphophilic compounds having surfactant properties and, like phytosterols, bind to cholesterol and bile acids.

Rao said that while nutritional interest in both phytosterols and saponins has focused on their cholesterol-lowering properties, some data suggest that these compounds may be anticarcinogens. In rats, β-sitosterol-supplemented diets (0.2% by weight) inhibit chemically induced colon cancer (*55*), and phytosterols reduce, in a dose-dependent fashion, cholic acid-induced colon cell proliferation and mitotic activity (*56*). Diets containing phytosterols at 1% by weight are well tolerated by experimental animals (*57*). Dietary saponins from soybeans and other sources have been shown to enhance immunity (*58,59*), are cytotoxic to Sarcoma 37 cells (*60*), inhibit DNA synthesis in tumor cells (*61*), decrease the growth of human epidermoid carcinoma cells (*62*) and human cervical carcinoma cells (*63*), and inhibit Epstein-Barr virus genome expression (*64*). Saponin-sup-

plemented diets (1% by weight), as is the case for the phytosterols, normalize abnormal colonic cell proliferative activity induced by carcinogens (Rao AV: unpublished observations).

Inositol Hexaphosphate

Ernst Graf discussed the rationale for the hypothesis in which inositol-1,2,3,4,5,6-hexaphosphate (IP_6), not fiber, is postulated to be responsible for the inverse correlation between the incidence of colon cancer and the consumption of fiber-rich foods (*65*). When the IP_6 content of cereals, fruits, and vegetables is considered, the international data suggest that there is a greater negative correlation between IP_6 and colon cancer incidence than between fiber and colon cancer incidence. IP_6 is found in a variety of plant foods, particularly cereals, but soybeans are an especially rich source, containing about 1.4% on a dry-weight basis (*66*).

Graf noted that most nutritional interest thus far has focused on the inhibitory effect of IP_6 on mineral absorption. IP_6 forms tight chelates with a variety of polyvalent metals such as calcium, zinc, and iron (*66*). However, he said, the ability to bind metal ions, particularly iron, may provide the basis for the anticarcinogenic effects of this compound. Graf commented that iron may be a key factor, via the Haber-Weiss reaction, in the production of hydroxyl radicals, which are postulated to play a role in the etiology of some cancers (*67*). IP_6 has been shown to limit the oxidant reactivity of transition metals (*66*), to inhibit lipid peroxidation (*67*), and to inhibit experimentally induced colon cancer (*68-73*). It has also been suggested that IP_6, through absorption following dephosphorylation to IP_3, could be an important second messenger involved in the regulation of cell differentiation (*73*).

Phytochemical Variation

James Duke discussed phytochemical variation in soybeans. Duke started by noting that there are over 10 000 named or numbered varieties of the common soybean *Glycine max L.* In these varieties, as one might expect, lies tremendous chemical variation. The genus *Glycine* was originally applied to a distant relative, now known as *Apios americana*, which is an edible root with more protein than is found in potato (*74*).

The isoflavone content of soybeans varies tremendously according to the plant part, variety, year harvested, and geographic location (*75*). Soybean hulls contain only relatively minor amounts of isoflavones, the majority of which occur in the hypocotyl, although one common isoflavone, genistein, is found primarily in the cotyledon (*75*). Equally significant are the reported differences in isoflavone content according to the varieties of soybeans and the year harvested. One study (*75*) reported a threefold variation in total isoflavone content among four varieties of soybeans, while a 30% variation was noted in a single variety of soybeans over a 4-year period. The content of individual isoflavones varied as much as 50%. Not surprisingly, location influences isoflavone content, even within fairly close geographical areas.

Duke noted that chemical variation is not limited to the isoflavones. In some instances as much as a fivefold variatio was found among different phenolic acids in soybeans, many o which have also been investigated as potential anticarcinogens.

Isoflavones in Plant Physiology

Renee Kosslak described the role of isoflavones in defense strategies utilized by plants. Plants produce a wide range of products or secondary metabolites thought to enhance their survival (*76*). The isoflavones daidzein and genistein are the major inducers of the nodulation genes in *Bradyrhizobium* bacteria, which form nodules on soybeans (*77*).

The genetic regulation of isoflavone synthesis in plants is not well understood, in part because of the limited number of appropriate mutants affecting this pathway (*78,79*). In soybeans, near-isogenic lines that differ in their root fluorescence are being examined to determine whether they are active in genetic regulation of isoflavone synthesis (*80*). (These differences in root fluorescence in soybeans were first observed in 1934.) There are five loci that affect root fluorescence (*80*), and although specific substances responsible for this property have not been identified, isoflavones are thought to be involved. Preliminary data indicate that the levels of daidzein, genistein, and coumestrol, which is also a phytoestrogen, were either reduced or absent in root extracts from three of the nonfluorescent isolines tested (Kosslak R: unpublished observations).

Kosslak suggested that if future research implicates isoflavones and/or phytoestrogens as important dietary factors in cancer prevention and if the demand for soybean specialty products materializes, it may be possible to manipulate levels of these compounds in soybeans, using root fluorescence as a marker.

Soybean Processing

Bernie Szuhaj briefly discussed soybean processing procedures (*81-83*). Solvent extraction is the primary method of producing soybean products today. Soybeans entering the plant are first cleaned, cracked, and dehulled. Then moisture is added so they can be "flaked," leaving a product that is 3% hypocotyl, 89% cotyledon, and 8% hulls. The oil is removed from the flakes by hexane, producing defatted flakes and soybean oil. From the defatted flakes come a variety of products with a protein content, on a dry-weight basis, that ranges from about 50% for soy flour and grits to about 60%-70% for protein concentrates and about 90% for protein isolates. The primary difference between soy protein concentrates and isolates is the larger percentage of carbohydrate in the soy protein concentrates. Many commercial doughnuts contain soy flour, and, in Europe and Asia, there is particular interest in the use of full-fat soy flours for baking.

Szuhaj noted that most soybean production today goes into animal feed, while the soy protein concentrates and isolates are marketed primarily for their multifunctional properties, such as emulsifying, gelling, fat-binding, texturizing, and dough forming. Soy products play a major role in the food chain. They are added to a wide variety of foods, from cereals to chili. Some

meat products, such as ground beef, contain up to 25% soy. These products have been used in the Armed Forces' canteens since 1983 and in the federal school lunch program.

Discussion

This workshop had two objectives: *1*) to evaluate the relationship between the risk of certain cancers and consumption of soybeans, products derived from soybeans, and/or specific components of soybeans and *2*) to recommend research initiatives aimed at clarifying this relationship. The consensus of the meeting was that there are sufficient data to justify studying the impact of soybean intake on cancer risk in humans.

There were three workshop recommendations. First, future dietary studies involving soybeans should be carried out using soy products rather than isolated compounds, since soybeans appear to contain several potential anticarcinogens. Additionally, because components of food interact, both negatively and positively, with each other, the potential benefit of soy products cannot be accurately predicted solely on the basis of the effects of individual soybean components. This does not, however, prohibit future use of isolated soybean components as chemopreventive agents in clinical trials. Second, standardized and improved analytical methods are needed so that the contents of all soy-based materials employed in soybean research, whether soybean fractions or soy products, can be accurately described. This methodology will allow for valid comparisons among studies. Third, basic research on the absorption, metabolism, and physiology of potential anticarcinogens in humans should be conducted. This research will likely help to determine the clinical relevancy of these compounds and to provide a basis for selecting specific soy products for use in future dietary studies.

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D7

Increasing use of soyfoods and their potential role in cancer prevention

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Abstract The United States produces approximately half of the world's soybeans. Although most of what is produced is used as animal feed, soy-protein products (eg, soy-protein flour, concentrates, and isolates) are used extensively by the food industry, primarily for their functional characteristics, such as emulsification. During the past decade, however, there has been a marked increase in the use of both traditional soyfoods, such as tofu and soymilk, and second-generation soyfoods, products which generally simulate familiar American dishes. Recently, attention has focused on the possible role of soybean consumption in reducing cancer risk. Soybeans contain, in relatively high concentrations, several compounds with demonstrated anticarcinogenic activity. Two of these compounds—protease inhibitors and phytic acid—have traditionally been viewed as antinutrients. The scientific community has begun to appreciate the potential importance of nonnutritive dietary compounds (phytochemicals) in foods such as soybeans. Dietitians need to become more aware of the phytochemical content of foods and the possible effect of phytochemicals on health and disease. *J Am Diet Assoc. 1991; 91:836-840.*

The United States produced more than $10 billion of soybeans in 1989, about half the world's total (1). Although most of the soybeans produced are used as animal feed (1), soy-protein products have been used extensively by the food industry since 1957 (2). Furthermore, during the past decade, there has been a marked increase in both the consumption of traditional soyfoods—such as tofu, soymilk, miso, and tempeh—and in the development of second-generation soyfoods (3,4). Second-generation soyfoods generally simulate traditional meat and dairy products, eg, soy hot dogs, soy sausage, and soy cheese.

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The increase in soyfood consumption, which is expected to continue throughout this decade, is attributed to a number of factors, including economics, health, ethics, and the environment. Recently, the potential role of soybeans in cancer prevention has received attention (5). As soyfoods continue to become part of the American diet, it is important for nutritionists and dietitians to familiarize themselves with soyfoods and their possible impact on health and disease.

Historical perspective

According to tradition, soybeans were one of five "sacred" crops named by Chinese emperor Sheng-nung nearly 5,000 years ago (6). The soybean has been widely consumed for hundreds of years throughout Asia. Soybeans were reportedly first brought to the United States in 1804 as ballast aboard a ship from China, but not until the 1890s was serious interest in soybeans expressed (7). World War I brought an increased interest in soybeans as a potential source of oil and of inexpensive, high-quality protein (7). In 1917, only 50,000 acres of soybeans were planted in the United States, but by 1931, this number had increased 70-fold (8). Throughout this century US soybean production has steadily increased; soybeans are now the second most important cash crop in the United States (9).

Soybeans and the US food supply

Soy-protein products

Soy protein products are grouped into three general categories: soy flour, soy-protein concentrates, and soy-protein isolates (2). The market for soy-protein products is estimated at around $200 million per year (3,4). More than 90% of the soybeans consumed by human beings in the United States, excluding soybeans used for soy oil, is in the form of soy-protein products (3). These products are made from defatted soybean flakes, range in protein content from about 50% to 90% and are added to a vast array of foods, primarily for their functional characteristics, such as emulsification (2). The per capita intake of soy-protein products in the United States has increased approximately 40% during the past 10 years, but soybean consumption is still less than 5 g/day per person (3). Thus, the nutrient contribution of soy-protein products for most individuals is negligible.

Two additional soy products used by the food industry are hydrolyzed vegetable protein and textured soy protein (2). Hydrolyzed vegetable protein is used as a whipping and flavoring agent and in the manufacture of soy sauce. Textured soy protein is frequently used as a ground beef extender, most notably by schools and the military. Textured soy protein is also used to produce meat analogues. The school lunch program uses an estimated 100 million lb textured soy protein annually (3).

Retail soyfoods sales

The US retail soyfood market has undergone considerable growth in recent years. In 1989 sales were more than $600 million, a fourfold increase since 1980 (3,4). During the past decade, approximately 200 new soyfood products have entered the market annually (3,4). After soy sauce, tofu is the biggest seller among all soyfoods, followed by second-generation soyfoods, soymilk, miso, soynuts, and tempeh (3).

Sales of soymilk and second-generation products are expected to increase substantially during the next 10 years (3). Soymilk consumption in particular is on the rise; sales have increased 40% during each of the last 2 years (3). By the year 2000, sales of soymilk are projected to increase sixfold and sales of second-generation products are expected to increase about fourfold (3). The increased consumption of soymilk has been attributed to improved taste, increased shelf-life through improved packaging, and lower cost resulting from increased domestic production.

The increasing number of second-generation soyfoods is particularly noteworthy. Since 1987, 552 new second-generation products have been developed; more than half of these contained tofu as their primary soy ingredient (3). Products range from main entrees—including frozen, shelf-stable, and refrigerated prepared foods—to meat alternatives such as tofu hot dogs and tempeh burgers, cheese alternatives, yogurts, and nondairy frozen desserts. In 1989, more than two thirds of all tofu and one third of all second-generation soyfoods were sold in supermarkets, a further indication that soyfoods are entering the mainstream (3). Nevertheless, with the exception of soy sauce, most soyfoods are consumed by a relatively small percentage of the population. For these individuals, soyfoods can be a major component of the diet.

Nutrient contribution of soyfoods

Whole soybeans are a good source of protein, fiber, calcium, iron, zinc, phosphorus, magnesium, thiamin, riboflavin, niacin, and folacin (10). Processing the whole soybean to make soyfoods affects the nutrient content of the resulting product (Table 1). Foods made from the whole soybean, such as tempeh, natto, and miso, are good sources of fiber and calcium, whereas soymilk and products made from soymilk, such as tofu and soy cheeses, are not. Calcium can be successfully added to soymilk (11); however, this has been done only sparingly, primarily in malted soy drinks. Calcium content of tofu varies quite notably. The values listed in Table 1 appear to be somewhat higher than one might surmise from a random, informal examination of commercially available products.

Sources of vitamin B-12 in soyfoods are an important consideration for certain individuals whose diet may be devoid of or contain only limited amounts of animal products. Some reports (10,12,13) indicate that fermented soyfoods, ie, miso and tempeh, contain vitamin B-12, either through design or by contamination, but other reports (14,15) suggest that most, if not all, of the vitamin B-12 in these products is in the form of analogues.

Soyfoods are relatively high in fat, but still may be lower in total fat than the foods they frequently replace, such as meats and cheeses. Soyfoods are certainly lower in saturated fat and cholesterol. Tofu ranges from about 35% to 50% fat on a caloric basis (silken tofu, which includes the whey, is generally lower in fat), whereas the fat content of soymilk varies depending on additional ingredients used. In contrast to skim milk, soymilks that are lower in fat are generally not produced by removing fat, but by adding carbohydrate, which is used as a sweetening or flavoring agent. Soybean oil is the only commonly consumed oil in this country that contains appreciable amounts of ω-3 fatty acids (α-linolenic acid). Consequently, soyfoods are a convenient means of obtaining ω-3 fatty acids from plant sources. However, hydrogenation of soy oil notably reduces linolenic acid content (16).

Soybeans and cancer risk

In 1929, soybean consumption was offered as a possible explanation for the greater stamina of northern Chinese compared with their rice-eating counterparts in the south (17). Throughout this century, soybeans have been the subject of considerable investigation covering a wide range of interests. Recently, the potential role of soybeans in cancer prevention has received attention. The contribution of soybeans to the diets of Oriental countries, such as Japan and China, has prompted some investigators to suggest that soybeans may contribute to the relatively low rates of breast and colon cancer in these countries. In June 1990, the National Cancer Institute held a workshop to examine this relationship (5).

Several compounds with anticarcinogenic activity are found in relatively high concentrations in soybeans. Among those thus far identified are isoflavones, protease inhibitors, phytic acid, saponins, phytosterols, and phenolic acids. Ironically, two of these compounds, protease inhibitors and phytic acid, have traditionally been viewed as antinutrients. Phytic acid effectively binds a variety of polyvalent metals, especially iron, and is considered to affect adversely the bioavailability of iron in soyfoods (18). The ability to chelate iron, however, may be responsible for the antioxidant and anticarcinogenic properties of phytic acid (19,20). Among the hypothesized anticarcinogens in soybeans, isoflavones and protease inhibitors are found in the highest concentrations relative to most other commonly consumed foods; consequently, in the discussion to follow, only these compounds will be examined, along with epidemiologic studies of soybean consumption and breast and colorectal cancer risk.

Experimental studies

Isoflavones. Barnes et al (21) showed that diets composed of as little as 5% (*wt/wt*) soybeans significantly inhibited chemically induced mammary cancer in rats. The study was particularly noteworthy because both raw and autoclaved soybeans were protective. This was an important observation because the protease inhibitors in

Table 1. Proximate composition and selected nutrient content of various soyfoods in common serving sizes and in 100-g edible portions (10)

component	miso[a]		natto[b]		okara[c]		roasted soybeans[d]		soymilk[e]		soy sauce (tamari)[f]		tempeh[g]		firm, raw tofu[h]		regular raw tofu[h]	
	½ c	100 g	½ c	100 g	½ c	100 g	½ c	100 g	½ c	100 g	1 tsp	100 g	½ c	100 g	¼ B[i]	100 g	¼ B	100 g
water (g)	57	41	48.4	55	50	82	1.7	2.0	112	93	11.9	66	45.6	55	57	70	98	85
kcal	284	206	187	212	47	77	405	471	39	33	11.0	60	165	199	118	145	88	76
protein (g)	16.3	11.8	15.6	17.7	2.0	3.2	30.3	35.2	3.3	2.8	1.9	10.5	15.7	19.0	12.8	15.8	9.4	8.1
lipid (g)	8.4	6.1	9.7	11.0	1.1	1.7	21.8	25.4	2.3	1.9	0.02	0.1	6.4	7.7	7.1	8.7	5.6	4.8
carbohydrate (g)	38.6	28	12.6	14.4	7.7	12.5	28.9	33.6	2.2	1.8	1.0	5.6	14.1	17.0	3.5	4.3	2.2	1.9
crude fiber (g)	3.4	2.5	1.4	1.6	2.5	4.1	4.0	4.6	13.2	1.1[j]	0.0	0.0	2.5	3.0	0.12	0.15	0.09	0.08
calcium (mg)	92	66	191	217	49	80	119	138	5	4	4	20	77	93	166[k]	205[k]	122[k]	105[k]
iron (mg)	3.8	2.7	7.6	8.6	0.79	1.3	3.4	3.9	0.7	0.6	0.43	2.38	1.9	2.3	8.48	10.5	6.2	5.4
zinc (mg)	4.6	3.3	2.7	3.0	...		2.7	3.1	0.27	0.23	0.08	0.43	1.5	1.8	1.27	1.57	0.93	0.8
thiamin (mg)	0.13	0.1	0.14	0.16	0.01	0.02	0.09	0.1	0.19	0.16	0.01	0.06	0.11	0.13	0.13	0.16	0.09	0.08
riboflavin (mg)	0.35	0.25	0.17	0.19	0.01	0.02	0.13	0.15	0.08	0.7	0.27	0.15	0.09	0.11	0.08	0.9	0.60	0.05
niacin (mg)	1.19	0.86	0.0	0.0	...		1.2	1.4	0.18	0.15	0.71	3.95	3.8	4.6	0.31	0.38	0.23	0.2
vitamin B-6 (mg)	0.3	0.22	...		...		0.18	0.21	0.05	0.4	0.36	0.2	0.25	0.3	0.08	0.09	0.06	0.05
folacin (μg)	45.5	33	...		...		182	211	1.8	1.5	3.3	18.2	43.2	52.0	23.7	29.3	17.4	15.0

[a]Cooked soybeans are dusted with *Aspergillus oryzae*, a bacterial starter, fermented, and pressed into a paste.
[b]Whole soybeans are steamed until soft, inoculated with *Bacillu natto*, fermented, and then commonly sold in 3- to 4-oz packages wrapped in straw.
[c]The pulp remaining after the soymilk is filtered; it is highly perishable.
[d]Whole soybeans are either dry- or oil-roasted in a pan or oven until crunchy.
[e]After overnight soaking, soybeans are pulverized, extensively cooked (to inactivate protease inhibitors), and then filtered resulting in a liquid soymilk.
[f]Insoluble dietary fiber as determined by the neutral detergent fiber method.
[g]Cooked soybeans are dusted with *Aspergillus oryzae*, shaped into koji nuggets, incubated, and mixed with salt and water to produce a mash called moromi. After aging, the moromi is pressed to yield a liquid and the oil is removed producing soy sauce.
[h]Cooked soybeans alone or with other grains are fermented with *Rhizopus oligosporus* producing a chunky-textured cake about ¾ inch thick.
[i]Soymilk is coagulated with a calcium or magnesium salt (nigari), the whey is discarded, and the curds are pressed to form a cohesive bond. The degree of pressing produces either soft, regular, or firm tofu. Calcium values refer to product made with nigari. The proximate composition of tofu shown here varies notably from recently adopted industry standards.
[j]B = block; one block refers to tofu 1¾ by 1¾ by 1⅝ in.
[k]No value reported.

soybeans, which are thought to be potent chemopreventive agents, are destroyed by autoclaving (22). The data of Barnes et al suggested that the isoflavones in soybeans were responsible for tumor inhibition. Soybeans are one of the few commonly consumed foods containing appreciable amounts of isoflavones (23).

A considerable amount of isoflavone research has been conducted, although very little is cancer related. For many years, isoflavones have been known to cause reproductive problems in agricultural animals (24), although similar effects have not been observed in populations consuming soyfoods.

In vitro data indicate that several isoflavones are weak estrogens, having perhaps as little as 1/100 of the affinity for the estrogen receptor as estradiol-17B (25,26). In vivo, isoflavones may act as antiestrogens in the presence of high levels of endogenous estrogens (25). This may occur because the isoflavones bind to the estrogen receptor without eliciting a substantial estrogenic response and competively inhibit the more potent estrogenic agonists from binding. The hypothesized antiestrogenic effect of isoflavones may help to reduce breast and perhaps endometrial and ovarian cancer, as these cancers are thought to be estrogen dependent (27). However, the effects of isoflavones may not be limited solely to hormone-related cancers.

In vitro, genistein, one of the isoflavones present in soybeans, inhibits tyrosine protein kinases, DNA topoisomerases, and S6 kinases (28-31). The activity of these enzymes is enhanced in oncogene-transformed cells (28-31). Consequently, isoflavones may have a role to play in the prevention of a wide range of cancers.

Human studies suggest that isoflavones may be physiologically important. Setchell et al (32) found that consumption of textured soy protein increased urinary isoflavone levels as much as 1,000-fold, although there were marked differences in isoflavone metabolism among the subjects in this study. Similarly, Adlercreutz et al (33) reported that urinary isoflavone levels were about 30 times higher in Japanese women consuming a traditional diet than in Finnish women.

Several studies have looked specifically at the estrogenic/antiestrogenic effects of soybeans. In postmenopausal women, soy intake appears to produce slight estrogenic effects (5,34); a recently completed study (A. Cassidy, S. A. Bingham, K. D. R. Setchell, unpublished data, 1991) however, found that in premenopausal women soy ingestion significantly lengthened the menstrual cycle. These studies suggest that isoflavones possess both antiestrogenic and estrogenic activity and, in premenopausal women, soy consumption influences hormonal patterns in a manner potentially protective against breast cancer.

Protease Inhibitors. For almost 50 years, the protease inhibitors in soybeans have been known to affect protein utilization adversely (35). Ironically, protease inhibitors were one of the first identified compounds to prevent

promotion of experimentally induced breast and colon cancer (22). The primary protease inhibitors in soybeans are the Kunitz trypsin inhibitor and the Bowman-Birk trypsin and chymotrypsin inhibitor (BBI). Most, but not all protease inhibitor activity is destroyed upon heating. Concern has been expressed over the potential harmful effects of consuming foods high in protease inhibitor content, particularly in the case of soy-based infant formulas (36). Raw soybean consumption causes pancreatic hypertrophy in rats (37). However, the response of the pancreas to protease inhibitors differs markedly among species (38); in populations consuming cooked soybean products no adverse effects have been observed.

Consumption of raw soybeans, which contain considerable protease inhibitor, markedly reduced experimentally induced breast cancer, although soy-fed rats weighed significantly less than the controls (39). Chymotrypsin inhibition is thought to be responsible for the anticancer activity of soybean protease inhibitors, although the inhibition of other proteases may also be important for inhibiting cancer (40). Soybean extracts containing BBI and/or pure BBI have been shown to inhibit experimental colon (41), lung (42), and oral cancer (43). Additionally, BBI inhibits x-irradiation–induced c-myc expression (44), decreases H_2O_2 formation by activated human polymorphonuclear leukocytes (45), and the soybean trypsin inhibitor suppressed the promotional effects of 12-0-tetradecanoylphorbol-13-acetate on transformation in C3H10T1/2 cells (40). Participants at a recent workshop expressed considerable enthusiasm for the development of protease inhibitors as chemopreventive agents (22).

Epidemiology. Much of the current thinking about diet-cancer hypotheses is based on studies comparing dietary intake among countries with the international variation in cancer incidence. These types of data are not available for soybean consumption and are probably not feasible to obtain. Most countries consume only minor amounts of soy products and/or soyfoods; only a handful, such as Taiwan, Japan, North and South Korea, Indonesia, and Hong Kong consume appreciable amounts (46). The epidemiologic data on soy and cancer consist primarily of case-control studies; case-control studies generally have been less supportive of diet-cancer associations.

Breast cancer. Despite the experimental data on isoflavones, little epidemiologic evidence suggests that soybeans lower breast cancer risk. Although a recent case control study (47) is strongly supportive of a protective effect of soy, Phillips (48) studied Seventh-day Adventists in the United States and found no difference in the consumption of vegetarian protein products between case and control subjects (48). However, vegetarian protein products were not exclusively soy, but included gluten-based products as well. No specific data on soy intake were recorded. Hirohata et al (49) found that soy was unrelated to breast cancer risk but, again, grams of fat derived from soy products rather than soy intake were recorded.

A very large prospective study involving 29 health districts and almost 143,000 women in Japan also found bean consumption to be unrelated to breast cancer mortality (50). No description of what constituted bean consumption was provided; however, from other dietary data it is apparent that bean consumption would primarily refer to soy products. In contrast, Nomura et al (51) found that soyfood consumption reduced breast cancer risk. However, findings were based on the dietary intake of husbands of subjects. Nomura and colleagues assumed that the dietary patterns of husbands and wives were similar. Spouses of controls consumed about 60% more miso soup ($P<.05$) and about 30% more tofu ($P<.16$) than did spouses of cases. However, Hiriayma (52) cautions that miso soup is frequently eaten in conjunction with vegetables, a factor that could be at least partially responsible for any protective effects of miso. Finally, Lee et al (47) in a case-control study of diet and breast cancer in Singapore found that soy protein in premenopausal women was protective ($P=.02$) and suggested that the phytoestrogens in soy may be responsible for this effect.

It may not be appropriate to evaluate soybeans on nutrient content alone; dietitians need to know about the nonnutritive dietary compounds, called phytochemicals, which may have anticarcinogenic effects

Colorectal cancer. Several studies suggest that soy consumption may lower colorectal cancer risk. Phillips (48) reported that colon cancer risk was reduced in individuals frequently consuming vegetarian protein products, although the effect was not significant (relative odds=0.4). Similarly, Watanabe et al (53) found that frequent consumption of soybeans and tofu markedly decreased both rectal and colon cancer risk (relative odds=0.14 for rectal cancer and 0.63 for colon cancer). Tuyns et al (54) also found that frequent consumption of soybeans decreased both rectal and colon cancer risk ($P<.0001$), and Poole (55) found that frequent consumption of tofu decreased colon cancer risk by approximately one half.

In contrast, Tajima and Tominaga (56), reported that consumption of miso soup significantly increased rectal cancer risk (relative risk, 2.05; $P<.05$) in Japanese subjects, although tofu only mildly increased risk. However, tofu was not associated with colon cancer risk in this study (55) and consumption of miso soup reduced colon cancer risk by one half.

Conclusions

Overall, the epidemiologic data suggest that soy consumption may lower colorectal cancer risk, whereas there is only moderate support for a role of soy in reducing breast cancer; however, relatively little work has been conducted in this area. Experimental work indicates that soy contains several potential anticarcinogens and that both estrogenic and antiestrogenic responses have been

observed in human beings consuming soy. Further investigation of the relationship between soy and cancer is needed.

Implications

As soyfoods continue to enter the American mainstream diet, their nutritional contributions will become increasingly important. However, it may no longer be appropriate to evaluate many foods, such as soybeans, solely on the basis of their nutrient content. The scientific community has begun to appreciate the effect of nonnutritive dietary components (phytochemicals) on health and disease, including diseases such as cancer. This places an additional burden on the nutrition community to become more aware of the phytochemical content of foods. Notably reducing the incidence of diet-related cancers will require major changes in the American diet. The extent to which soyfoods may help to achieve this goal remains to be seen.

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Catheterisation: your urethra in their hands

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Br Med J 1990;301:905

The emphasis in undergraduate medical education is often on the theoretical aspects of medicine rather than the practical aspects. Practical procedures are commonly taught informally, the teaching being passed from one junior to the next.[1] The philosophy is of "See one, do one, teach one." Urethral catheterisation is a procedure that requires a certain amount of skill, knowledge, and experience and is not without complication,[2-4] yet it is usually delegated to the most junior and inexperienced medical staff, the junior house officers.

Subjects, methods, and results

To assess the level of competence at catheterisation among junior medical staff house officers at this hospital were interviewed with a structured questionnaire, covering three aspects of the procedure: the degree of undergraduate and postgraduate instruction, the practical and theoretical aspects of catheterisation, and, finally, problems and complications encountered.

Thirty junior house officers (graduates of five medical schools) were interviewed. Eighteen were male and 12 were female. The replies to the questionnaire showed that none of the interviewees had received any formal instruction regarding any aspect of urethral catheterisation as an undergraduate. Practical postgraduate instruction in 24 was limited to supervision of a single catheterisation, and four subjects were unsupervised. Although those interviewed had performed a mean of 28 (range 6-100) catheterisations in male patients, only four of them had catheterised female patients.

Despite the large number of procedures performed there was appreciable ignorance of the practical and theoretical aspects of catheterisation. Twenty five interviewees were unaware of the availability of short term and long term catheters or of the duration for which they may be safely left without being changed. Three interviewees simply used the catheter that was provided by the nursing staff, and one did not know that different sizes existed.

Twenty eight interviewees initially used force when meeting resistance to the passage of the catheter, and 13 stated that the development of fresh urethral bleeding would not deter them from a further attempt at catheterisation. Eighteen were happy to attempt catheterisation in a patient who had a known urethral stricture. Five interviewees were unaware of the difference between a phimosis and paraphimosis.

Despite the lack of formal tuition all had developed what seemed to be a satisfactory aseptic technique. None, however, was aware of the nature of the antiseptic fluid or the strength of the local anaesthetic gel, but simply used what was provided by the nursing staff.

Nineteen of the interviewees had encountered bleeding and six had had patients in whom a paraphimosis had developed after catheterisation. A particularly disturbing finding was that, although 14 interviewees had requested help from senior medical staff, seven were reluctant to seek advice, because of their impression that difficulties with catheterisation were not worthy of disturbing senior staff. Eight of the 12 female medical staff had encountered problems with male patients becoming sexually excited during the procedure.

Discussion

The results of our survey suggest that the technique of urethral catheterisation is poorly taught, and in the light of these results we are preparing a short teaching video to be shown to every house officer at the start of their preregistration post.

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Oestrogenic effects of plant foods in postmenopausal women

Gisela Wilcox, Mark L Wahlqvist, Henry G Burger, Gabriele Medley

Crops grown as animal pasture are known to have oestrogenic activity,[1] and some foods contain potential oestrogenic analogues such as the isoflavonoids (isoflavones and coumestans), lignans, and resorcylic acid lactones,[2] which may be activated or inactivated.[3] We studied the effect of three foods reported to induce vaginal oestrus in laboratory animals[1] in postmenopausal women not taking oestrogen replacement therapy.

Subjects, methods, and results

We studied 25 postmenopausal women who were non-smokers, in good general health, and taking no drugs known to affect oestrogen state (mean age 59 (range 51-70); body mass index 24·4 (range 18·7-31·6) kg/m^2; years after menopause 8·1 (range 1-20)). The protocol was a latin square design with a two week run in period and a six week experimental period. The women recorded their normal diet for 14 days and were asked to repeat the fortnightly diet throughout the study. During the experimental period the diet was

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supplemented with soya flour (45 g daily), red clover sprouts (10 g dry seed daily), and linseed (25 g daily), each for two weeks in turn. To check compliance the women returned residual food. Blood samples were taken weekly and lateral wall vaginal smears taken fortnightly and at follow up two and eight weeks after supplementation finished. Analysis was on intention to treat, but 23 women completed the study.

We examined the dependent variables vaginal cell maturation and serum concentrations of luteinising hormone and follicle stimulating hormone. The cumulative effects of the three foods at six weeks were compared with baseline by the paired *t* test, as were the residual effects, two and eight weeks after the last food supplement. We found significant differences in vaginal cytology after six weeks' supplementation ($p<0.01$, 95% confidence interval 6·0 to 17·6), which persisted for two weeks after treatment ($p<0.02$), but cytology returned to baseline after eight weeks (table).

Mean (SE) values for oestrogenic indicators in postmenopausal women consuming phyto-oestrogens

Week		Maturation value	Luteinising hormone (IU/l)	Follicle stimulating hormone (IU/l)
1			45·7 (3·1)	58·7 (2·9)
2		30·8 (4·5)	46·6 (3·4)	58·7 (3·0)
3	Food supplementation		50·8 (8·5)	57·4 (2·9)
4	Food supplementation	35·0 (5·1)	46·0 (3·6)	57·3 (2·9)
5	Food supplementation		46·2 (3·3)	57·7 (3·0)
6	Food supplementation	39·6 (5·3)	42·9 (3·2)	54·3 (2·9)
7	Food supplementation		43·6 (3·3)	56·4 (2·8)
8	Food supplementation	45·4 (3·6)	44·6 (3·3)	54·8 (2·4)
9			44·9 (3·5)	57·9 (2·8)
10		43·6 (4·7)	44·9 (3·5)	57·5 (2·7)
16		33·7 (5·5)		

The maturation value significantly increased after soya flour ($p<0.05$) and linseed ($p<0.02$) but not after red clover sprouts ($p=0.11$).

All women had concentrations of follicle stimulating hormone and luteinising hormone greater than those in the premenopausal range of 2-8 IU/l and 6-13 IU/l respectively. There was a cumulative effect on serum concentrations of follicle stimulating hormone ($p<0.05$) but not on luteinising hormone over the six week supplementation period. Individual two week food supplements had no measurable effects on either hormone.

In seven women with the most pronounced changes in vaginal cytology we measured serum oestradiol concentrations weekly. Baseline concentrations were <70 pmol/l in all but one woman, who was retained as the study was based on intention to treat. There were no appreciable changes in body weight during the study.

Comment

We aimed to consider whether phyto-oestrogens were of consequence in human nutrition. Our study gives some indication of the recovery time from any possible effect of treatment and also provides further evidence of causality. Vaginal maturation is a sensitive and specific indicator of oestrogenicity. Follicle stimulating hormone is less sensitive to weak oestrogenic compounds such as phyto-oestrogens. Weak oestrogenic compounds may sometimes act as antioestrogens, which may affect their usefulness as sources of oestrogenic activity. Conversely, tamoxifen, an antioestrogen, can have oestrogenic effects on vaginal cytology.[5]

Patterns of food intake may modulate the severity of the menopause as it is an oestrogen deficiency state. Up to half of the diet of some populations may comprise foods containing phyto-oestrogens, whereas in our study such foods comprised only about 10% of energy intake for a fairly short time. Whether menopausal symptoms differ in such populations would be worth investigation.

We thank our statistical adviser, Steve Forbes, from the department of social and preventive medicine, Monash University.

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Inadvertent duplicate publication

Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears

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The *BMJ* regrets that much of the material in the above article (30 June 1990, *p* 1690) was substantially the same as that published previously in *Contemporary Reviews in Obstetrics and Gynaecology* (Redman CWE, Buxton EJ, Cullimore J, Luesley DM. Loop diathermy excision of the cervical transformation zone in the management of cervical intraepithelial neoplasia. 1990;2:53-8). The authors did not tell us this when the article was submitted, their article did not contain any reference to the earlier paper, and all authors signed our copyright form, which states, among other things, that "papers are accepted on condition that they have not been published by any other journal."

We regret this inadvertent duplicate publication, for which the authors hold sole responsibility, and which is in violation of our Instructions to Authors and internationally agreed guidelines.

Correction

Incidence of peptic ulcer disease in Gothenburg, 1985

An editorial error occurred in this paper by Dr Ivy-Mai Schöön and others (1989;299:1132). The y axis of figure 1 should read 0, 5, 10, 15, and 20 and not 0, 0·5, 1·0, 1·5, and 2·0 as published.

Herbs used

Internally

Echinacea *Golden seal* *Raspberry leaf* *Thyme*

Douche

Equal parts *Calendula* and *Comfrey* or *Calendula* and *Golden seal* with 3 drops *Sandalwood* or *Tea-tree oil.*

Tea-tree oil suppositories are now available.

From the kitchen

Dilute *Apple cider vinegar* douche

Supplements

Vitamin C and bioflavonoids
Garlic capsules (6 per day in divided doses after food)

Tissue salt

Kali Mur

MENOPAUSE

General

Description

The end of menstruation.

Cause

A normal occurrence, usually taking place between the age of 45–55 years, although occasionally earlier. When the ovaries stop producing ova (eggs) the menopause will be finished. Women who have partial hysterectomies in which their ovaries are not removed, will experience menospause when their ovaries stop producing. Many women go through the transition easily.

The ovaries are responsible for producing most of the oestrogen and progesterone. These hormones can be produced in other glands, such as the adrenals, which explains why some women do not experience the symptoms to the same extent. As our hormonal system works on a feedback system, if pharmaceutical therapy is used the glands will not be stimulated to function.

Herbs used

There are a number of herbs and foods which contain small amounts of plant oestrogens and progesterone, or substances which function in a similar way.

Oestrogenic plants

Alfalfa – best used as sprouts
Aniseed *Fennel* *Liquorice* – not the confectionery
Parsley – best used fresh; a handful daily
Red clover – best used as sprouts
Sage – as a cold tea; see *Hot Flushes*, see below
Soya beans – best used as sprouts. Difficult to sprout in cold weather.

Plants with progesterone precursors

Fenugreek – sprouts are more effective
Sarsaparilla *Wild yam*

Include about one cup of sprouts per day in your diet. Professional herbalists use some other herbs which are also in these categories but they are rarely available to the general public. A Chinese herb called dong quai is commonly recommended for menopausal problems and is stocked by some health food stores.

Other herbs are also useful and these will be shown with each of the symptoms.

Hot Flushes

Description

A sudden, hot feeling, usually starting in the chest and going up to the face. Sometimes the skin gets quite red. This may be followed by heavy sweating, so a change of clothes or a warm jacket should be kept handy.

Cause

The flushing is said to be the result of the effort of the controlling centre in the brain to stimulate female hormone production but as the ovaries are no longer effective, all that is achieved is an increase in circulation which manifests as heat.

Herbs especially recommended

Sage – use a cold infusion, as explained in Chapter 4, page 140.
Alfalfa and *Red clover* sprouts
Liquorice (not the confectionery) *Ginseng*

Supplements

Vitamin E

Tissue salts

Kali Sulph

Taken from:
Beckham N, 1988, The Family Guide to Natural Therapies, Greenhouse Publications. Richmond.

Depression and Lethargy

It is commonly believed that women who lead useful, interesting lives do not apparently exhibit the same degree of symptoms. Boredom is a common cause of depression. Don't listen to people who tell you 'it's normal to be unwell at your age':

Herbs especially recommended

Chilli – only if there is no flushing
Ginseng *Liquorice* *Oats*

All the oestrogenic and progestogenic herbs except hops.

Other

A number of community health centres run programmes for menopausal women.
Find some new interests – you probably have another thirty years to go!

Tissue salt

Nat Mur

Nervous Agitation, Anxiety

Herbs respecially recommended

Hops *Linden* *Motherwort*
(Refer also to Chapter 8)

Other

Avoid the use of tranquillisers and similar drugs; there are already over 8 000 000 of these prescribed in Australia each year.
Learn yoga or relaxation and practise for 15–20 minutes daily. Don't drink tea, coffee, cola, and reduce alcohol to 1 glass daily or none.

Tissue salt

Kali Phos

Osteoporosis

Description

This is the most serious change associated with the menopause and the most common reason for the prescribing of Hormone Replacement Therapy. The density of the bones throughout the body starts to decline (commonly known as brittle bones) which is why elderly women often fracture easily and have difficulty healing.

Cause

As the levels of oestrogen fall, there is a corresponding loss of calcium from the bones. In severe cases, a 70 year old woman may have lost 50 per cent of her total bone mass. Excessive coffee drinking interferes with calcium absorption. Anorexic women of any age suffer bone loss and a prolonged low calorie diet can have the same effect. Excess protein also causes calcium loss.

This information relates to most of the so-called developed countries. In certain countries there is no known osteoporosis and in others there is more in males than females which indicates that the problem is not solely linked to female hormone changes.

Herbs especially recommended

All the herbs in the oestrogen list and *Horsetail.*

Diet

Calcium supplementation

There are hundreds of published scientific research papers on calcium and its relationship to osteoporosis. A number of researchers now recommend that the dietary intake of calcium for adult females should be between 1000–1500 mg. Most practitioners agree that the majority of middle aged women would not eat this quantity in their normal diet, so there is a fairly clear indication that supplementation is beneficial. The form this supplementation should take is debatable. There are many tablet formulas which incorporate calcium, not all researchers agree on the most appropriate dosage and, most importantly, not all people absorb calcium to the same degree. There is also some conflict relating to the correct method of measuring bone density and what other nutrients should or should not be taken with calcium.

Some studies indicate that female vegetarians who eat dairy products and eggs have less bone mineral loss. In certain areas of the world, where protein intake is extremely low, post-menopausal osteoporosis is unknown; however, it would not be intelligent to become protein deficient in order to preserve your bones. Another report indicated that a significant percentage of osteoporotic females are intolerant to dairy products.

Because there is so much scientific and individual variation, I suggest the following:

All adult females up to 35 years of age

One multi-vitamin supplement per day after breakfast.

Herbs used

Evening primrose oil *Motherwort*

Other herbs which may be helpful are indicated in Chapter 8.

Diet

Safflower oil – 2 teaspoons per day if not taking the *Evening primrose oil.*

Small frequent meals, increase protein intake if on a low protein diet.

Supplements

Spirulina – 2 tablets three times a day between meals.

PMT-D

Description

Depression, forgetfulness, crying, confusion, insomnia.

Causes

High progesterone, low oestrogen, high androgens. Occasionally lead toxicity.

Herbs used

Oestrogenic herbs

Alfalfa *Aniseed* *Fennel* *Liquorice* *Parsley* *Red clover* *Sage* *Soya beans*

The intake of these should be high just before the start of the PMT period up to menstruation.

Other herbs helpful in depression

Ginseng *Ginger* *Oats*

From the kitchen

Rosemary

Diet

Supplements

B complex with additional B1 and B3 *Glutamine*

PMT-H

Description

Fluid retention, weight gain, sore breasts, abdominal bloating, fatigue. Possible allergies, high adrenal hormones.

Herbs used

All the oestrogenic herbs **except liquorice**

Herbal diuretics such as

Corn silk *Dandelion* *Horsetail*

CAUTION Do not use Ginseng.

From the kitchen

Celery juice *Cucumber juice*

Diet

Reduce salt, coffee, tea, smoking.

Supplements

B complex with additional B6 *Vitamin E* *Potassium* *Celery and juniper tablets*

PMT-P

Description

Generalised aches and pains.

Causes

Possible magnesium deficiency or calcium excess.

Diet

Supplements

Magnesium

PMT – with migraine type headache

Description

A throbbing, vascular headache affecting one side of the head, often accompanied by sensitivity to light and nausea. Usually lasts all day.

Cause

Believed to relate to low oestrogen, high progesterone.
Might also be an allergy or circulatory problem.

Herbs used

Oestrogenic herbs *Feverfew*

Other

Hot foot baths

PMT – with acne

Description

Outbreak of pimples coinciding with the premenstrual time (commonly 5–12 days before periods).

Herbal Help to Avoid Menopause Symptoms

by Nancy Beckham

The aim of this article is to provide information on non-harmful ways of overcoming the problems of menopause.

The information given may also be applicable to women who already have osteoporosis or for younger women who have had their ovaries removed, however these two categories of women should seek professional guidance.

SOME STATISTICS

Twenty-five per cent of women in the 45-55 age range have no menopausal symptoms. Of the 75 per cent who have problems, the following is a breakdown of the symptoms:

Flushing and sweats	80%
Lethargy	70%
Nervous problems such as anxiety, depression, irritability	70%
Reduced sex drive	65%
Insomnia	60%

Other symptoms include hair and skin changes, poor memory and lack of concentration, headaches, dry vagina, pain during intercourse, loss of confidence, loss of femininity and urinary symptoms. Of course, not all of these are necessarily linked to low oestrogen levels and could be related to dietary and lifestyle factors and the 'normal' aging process. After middle age, men also find they have less energy, a lower sex drive and generally sleep less.

Osteoporosis is the most serious problem associated with menopause because as much as 50 per cent of total bone mass may be lost by the time a woman reaches 70 years of age, which means that bones can fracture easily and healing may be prolonged. This disease does not affect all regions – it is rare in African Negroes and there are areas where it affects more men than women. In Australia, it is estimated that about 25 per cent of post-menopausal women have osteoporosis. I will deal with this in detail next issue.

What happens when the ovaries stop functioning?

The major factor is the lowered production of oestrogen. However, this hormone can be produced in other glands, such as the adrenals, but obviously, in many women, this does not occur quickly enough or in sufficient quantities. Basically, the hormonal system works on a feedback system; when the circulating levels are high, a chemical 'messenger' instructs our endocrine system not to produce any more of that particular hormone. Obviously, if we flood our system with a hormonal drug, the messages to our endocrine system will be to stop production. This may explain why some women do not menstruate for varying periods when they stop taking the Pill.

Most menopausal-age women will need to give their bodies as much assistance as possible so that sufficient oestrogen is produced to offset flushing and other symptoms. In nearly every case, this apparently happens over a period of time as the obvious symptoms gradually lessen and disappear. This added function of the adrenals may partly explain why some women have difficulty handling stress at menopause.

The controversial topic of hormonal replacement therapy will be discussed in detail later but in view of this feedback mechanism, it may not be wise to completely dampen the corrective biological function which already exists.

I am not suggesting that we can avoid the inevitability of aging, but I can't accept that whatever power 'designed' us also programmed that we were predestined to suffer a range of serious problems after middle age. We must be doing something wrong or there must be non-harmful methods of preventing the symptoms.

OESTROGENS IN FOODS AND HERBS AND HOW TO USE THEM

Since the 1920s over 50 different species of plant have been found to contain oestrogenic substances. Most of the published research papers relate to the effects on animals, particularly in respect of clovers and alfalfa (lucerne) causing infertility in farm animals. It so happens that a number of these plants have been used by herbalists over the centuries and this 'tested' use on humans has verified the hormonal effect. The tiny quantities of oestrogens in plants are extremely weak compared to pharmaceutical hormones but many women alleviate symptoms through sensible dietary changes.

Some of these oestrogen-containing plants are:

Alfalfa

The sprouts are particularly recommended as they have the added advantage of being very low in calories, readily available in shops or you can make your own, palatable in salads or sandwiches, mildly alkaline and rich in nutrients, especially calcium and potassium.

Alfalfa sprouts are somewhat controversial at the moment as the Gerson Institute in Mexico has reported that they suppress the immune system and aggravate conditions such as rheumatoid arthritis and systemic lupus erythematosus (SLE). The origin of this report was that two women had seemingly reactivated SLE following the ingestion of 10 and 15 alfalfa tablets per day. A particular constituent, L-canavanine, was extracted from alfalfa and when this isolated extract was given to susceptible animals, SLE was reactivated.

My own view is that, as SLE is a condition which has relative periods of aggravation and remission, it would be difficult to 'blame' one particular dietary item. Over 25 pharmaceuticals exacerbate the disease, isolated extracts of plants are in the nature of drugs and one would therefore expect side-effects and, most importantly, if this type of criterion were applied to almost any edible food, there would be very little left for us to eat. However, it may be prudent for sufferers of SLE to avoid alfalfa, all sprouted seeds and legumes, such as lentils, because these also contain the suspected irritant.

Red Clover

This is commonly sold as a herbal tea but I suggest you buy the seeds and sprout them. Please do not pick the clover yourself because the medicinal species, called *Trifolium pratense*, is difficult to distinguish from some non-edible clovers. Red clover is also used by professional herbalists for skin and respiratory conditions.

Sage

The common garden sage or red sage is used. It is better to grow your own but sage can be difficult to cultivate, mainly because it prefers a light, well-drained soil.

Sage has been used for centuries for excess sweating and heat, and scientific research has confirmed its oestrogen content. The best way to prepare it as a remedy for flushing is to soak two

tablespoons of finely chopped fresh leaves (or one tablespoon dried) in 500 mL tepid water with the juice of a lemon. Leave it stand in a covered jar overnight. Strain and keep in the fridge. In severe cases you would drink the whole quantity throughout the day; where the symptoms are relatively minor, then the 500 mL could be spread over two or three days. To make it more palatable, you could mix it with a fruit or vegetable juice, or add in some crushed fennel or aniseed. The high dose may need to be used for up to four weeks, then it could be gradually reduced to a cup per day.

The old sage you have had in your cupboard since two Christmases ago probably no longer retains any therapeutic properties; good-quality dried sage will still have a reasonably good colour and its characteristic strong odour.

Parsley

This common culinary herb has oestrogen-like activity and I would suggest a handful per day; it may not be wise to use larger quantities because of the myristicin and apiol content.

Aniseed

Use the crushed seeds as a herbal tea or in cooking, for example in home-made bread. The seeds could also be added to apple cider vinegar and used in salad dressings. Finely chopped fresh leaves can be added to salads, steamed vegetables and soups. Aniseed is also helpful for minor digestive problems and coughs.

Fennel

The seeds and finely chopped fresh leaves can be used in a similar way to aniseed. There is one species of fennel (Florence) which develops a bulb-like base and this may be used like celery or lightly steamed. Some greengrocers call it aniseed root. Wild fennel is a common weed and, although the seeds and leaves could be used, this plant is often contaminated with environmental pollutants.

Similar culinary herbs, such as dill and caraway, probably contain mild oestrogen-like substances.

Hops

Some health-food stores sell dried hops. It is somewhat bitter, which may also stimulate the digestive function, but the tea should be made quite weak otherwise it is not very palatable. An important feature of hops is that it has a sedative function and for this reason herbal extracts of hops are not used by professional herbalists where there is depression. Many people find that hops helps with insomnia – a herbal pillow using dried hops can be quite beneficial.

The hormonal content of hops has been verified; females harvesting it have altered menstrual periods solely from external contact. I am not sure whether or not beer, after all the processing, would retain any oestrogenic properties.

Soya Beans

Sprouts are the best way to have these, particularly as the sprouting dramatically increases the oestrogen content. However, they are quite difficult to sprout because they go mouldy and smelly if not washed and drained thoroughly and often. They are amazingly tasty but wait until you have learned to sprout alfalfa and mung beans before trying them. I add soya bean sprouts to salads or use them to thicken soups and casseroles.

Dried soya beans need to be soaked and cooked for a long time and they are probably best used in soups and casseroles but there are many ways of preparing them to make them more appetising. They are cheap, an excellent protein when combined with a grain and have other benefits, such as being protective against atherosclerosis.

If you don't normally eat dried beans then you must start with small quantities, soaked overnight and very well cooked, otherwise you will probably have severe abdominal colic and flatulence. This is partly because certain enzymes have to be activated to handle such foods and your digestive system needs time to adjust.

Soya beans are also a leguminous plant so, theoretically, could have the same cautions as indicated under alfalfa.

Dried red beans and common green beans are also mildly oestrogenic so could be included in the diet on a regular basis.

There is some evidence that all young sprouts, including sprouted grains and legumes, have oestrogenic properties and, as sprouts are cheap, pesticide and chemical free, rich in nutrients and low in calories, I recommend that you learn how to do your own and have at least one cup per day if you are a menopause-age female. You can buy small paperback books giving you basic instructions for sprouting and use jars, so the starting equipment is not expensive.

Some words of warning: When using seeds to sprout, never use those that are intended for agricultural purposes because they would have been treated with fungicides or other chemicals which are potentially dangerous.

Fenugreek

Contains precursors of progesterone, another female hormone commonly deficient in menopausal women. Unfortunately, the curry-like smell is readily excreted through the skin but this is not so noticeable if the seeds are sprouted.

There are other herbal and naturopathic remedies for menopausal problems but these are not normally available at retail outlets so you would need to visit a practitioner to obtain these. As with most health problems, there are mild symptoms which require no treatment or simple home remedies; then there are other instances where professional naturopathic advice is helpful and appropriate; and, finally, there are severe cases which require medical diagnosis and treatment.

OTHER SUGGESTIONS

Potassium sulphate, used in the form of tissue salts, may be helpful for flushing. Use the dosage on the label, but take double the dose for the first week.

Vitamin E has also alleviated some cases of flushing; furthermore, a study on rats showed that a vitamin E deficiency leads to lower bone weight. As this vitamin has benefits to the cardiovascular system, a supplement of 500 iu per day would do no harm and may give marked benefits.

Cigarette smoking tends to bring on early menopause and is not recommended for this and other well-publicised reasons.

Low-caloric diets are not recommended for a number of reasons which are given later, but one important factor is that fat cells are able to convert hormones from the adrenal glands into oestrogen. Although modern women, including myself, don't want or need to be obese, it may be that 'nature' intended us to carry more weight as we age.

Readers may be interested in a few snippets from some of the research material which I have collected:

Journal of Food Protection, Vol. 42, July 1979, states that 'human exposure to dietary oestrogens is below physiological levels... but the possibility of metabolic alterations to more or less active forms should not be ignored since effects of this kind have been demonstrated in experimental animals.'

Oestrogenic Constituents of Forage Plants, E.M. Bickoff, Review Series 1/1968, published by the Commonwealth Bureau of Pastures and Field Crops, Hurley, Berkshire, reports that 'the classical infertility syndrome in ewes is associated with the cumulative effects of exposure to oestrogenic feeding for six months or longer, but short-term exposure has also caused reproductive disturbances.'

The effect of oestrogenic plant substances is judged by changes in the anatomy of animals, for example increased uterine and ovarian weight, teat length and thicker vaginal skin.

A particularly interesting piece of research has shown that genistein, a weak plant oestrogen, is able to displace oestradiol from receptors in the tissues which could explain why some herbal

Continued on next page

Herbal Help to Avoid Menopause Symptoms

remedies are traditionally used for 'balancing' hormones.

Both the liver and the kidneys have a capacity for converting and deactivating different types of oestrogens; there are also other regulatory mechanisms, such as the prostaglandins.

HORMONE REPLACEMENT THERAPY

Although mainstream medical opinion supports hormone replacement therapy, it is somewhat controversial. Few disagree with the fact that it prevents the worsening of osteoporosis in postmenopausal females but the main disadvantage is in the potential side-effects. The current scientific thinking is that if both oestrogen and progestogen are taken together there is less risk of cancer. I am using Depo-Provera and Premarin as examples because a lady I saw recently had been prescribed these for menopausal flushing and she had written to the two manufacturers for information. The manufacturer of Premarin sent back 22 pages of reports and a book, all giving glowing testimonials and other information but only a few fragments about risks. The manufacturer of Depo-Provera sent a copy of the official package insert, with all the contraindications and side-effects, along with a letter which stated that 'as adjunct to cyclic oestrogen therapy (including Premarin) Depo-Provera is not recommended but it is still definitely used by medical practitioners'.

Depo-Provera

The contraindications and warnings for this drug include thromboembolic disorders (clotting), cerebral apoplexy (stroke), impaired liver function, undiagnosed vaginal bleeding and cerebrovascular (heart and circulatory) disorders. 'In cases of partial or complete loss of vision, sudden onset of proptosis (displacement of an organ), double vision, migraine associated with retinal vascular lesions, medication should be withdrawn.' The drug caused malignant breast nodules in animals. Other problems include fluid retention, breakthrough bleeding and depression. It is not approved for contraception because of unresolved questions relating to its safety for this purpose. Clinically, it is said that the drug is well tolerated although animal studies show that it possesses adrenocorticoid activity and female masculinisation.

Premarin

This drug is 'probably effective for oestrogen deficiency-induced osteoporosis only when used in conjunction with other important measures such as diet, calcium, physiotherapy and good general health-promoting measures'. The contraindications and cautions include impaired liver function, breast cancer (with some exceptions), thromboembolic disorders, undiagnosed abnormal genital bleeding and pregnancy. It should not be given to women with recurrent chronic mastitis and abnormal mammograms. It should only be prescribed following a complete breast and pelvis examination. Because the body produces variable amounts of oestrogen, relative overdosage may occur which could lead to uterine bleeding, painful, swollen breasts and fluid retention. Drug oestrogens need to be used with care in cases of epilepsy, migraine, asthma, heart or kidney disease. Side-effects include nausea, abdominal cramps and bloating, breast tenderness, changes in body weight, allergic rash and gallbladder complications.

When the two are prescribed together, there is often a monthly bleed.

If you are one of those people who believe that 'it would not be allowed by the government if there were risks involved', I suggest you read the information for yourself in *Mims Annual* which is available at most libraries.

A report in the *New England Journal of Medicine*, 19 June 1986, states that 'oral administration of oestrogens is inefficient, produces a non-physiologic pattern of breakdown products and increases undesirable levels of certain liver proteins'. The article examines the use of transdermal oestrogen therapy (skin patches) which would provide levels equivalent to those produced naturally. However, according to *Modern Medicine in Australia*, August 1986, transdermal oestrogen does not prevent osteoporosis.

Mainstream medical reports recommend oestrogen, used in conjunction with progesterone, as being the most effective therapy for preventing fractures, and a number of international experts suggest that all women should be considered probable victims and that hormone replacement therapy should begin soon after menopause in women, unless there are specific contraindications. The reasons for this are that at this stage there are no practical methods of clearly preselecting those at risk and tests show that it is the only therapy that clearly prevents further bone deterioration.

The critics point out that although adding progesterone to the therapy probably reduces the risk of endometrial cancer, there is insufficient data about the long-term safety or benefits – it only treats the symptoms and is unlikely to help the body re-establish its own state of internal harmony.

A pamphlet issued by the NSW Department of Health states that you should 'think carefully about whether or not you want hormone replacement therapy'. Some of my patients have understood from their medical consultations that hormone replacement therapy prevents cancer and cardiovascular disease, which is not true. Patients who are under this impression should discuss this matter with their practitioners.

The fact that a substance can prevent further bone breakdown does not necessarily mean that lack of it caused the problem, just as Valium helps you sleep but lack of it did not cause your insomnia.

In any event, most experts agree that there are clearly individuals who should not undertake hormonal replacement therapy and such people can use the non-harmful suggestions in this article.

No one enjoys being wrinkled and cranky but it is generally conceded that hormonal replacement therapy is not appropriate for cosmetic and emotional purposes.

Next issue: Osteoporosis and calcium requirements.

Nancy Beckham is the author of The Family Guide to Natural Therapies, *Greenhouse Publications, recommended retail price, $24.95.*

COMMONWEALTH OF AUSTRALIA

IN THE MATTER OF Australian Patent Application Serial No. 683838 in the name of NOVOGEN RESEARCH PTY LTD
-and-
IN THE MATTER OF Opposition thereto by MEDIHERB PTY LTD (A.C.N. 006 454 717)

STATUTORY DECLARATION

I, Nancy BECKHAM of 1 River Road, Oatley, New South Wales, 2223, Australia, do solemnly and sincerely declare as follows:-

1. I am a practicing natural therapist and have been treating patients since the early 1980's. As a natural therapies student in 1978 I became interested in phyto-oestrogens. Phyto-oestrogens are plant hormones which have been found to exert oestrogenic or anti-oestrogenic effects on animals and humans. My interest in this area was initiated by reports in the scientific literature that sheep when grazing on clover became infertile. This infertility was attributed to the presence of isoflavonic phyto-oestrogens which were known to be present in the clover. I reasoned at that time that if the large quantities that animals consumed gave an undesirable side effect because of the isoflavonic phyto-oestrogens, then the smaller quantities that humans could reasonably consume would give beneficial effects, especially in women with low oestrogen levels. The preponderance of accumulated scientific data, much of which was published before 1992, which describes the effect of a diet high in phyto-oestrogens on humans, has confirmed my initial suppositions. In 1980 I began testing the effect of phyto-oestrogens on menopausal females and the benefits of this treatment were clearly verified on many patients. By 1985, it was clear from my clinical experience and research that phyto-oestrogens were valuable therapeutically. Between then and 1992, I began using phyto-oestrogenic plants in my clinic, not only for menopausal symptoms but also for various conditions relating to imbalances of female hormones (endometriosis, fibrocystic breast disease and the like), lowering cholesterol, treating prostate problems and as an adjunct therapy for cancer and osteoporosis. The phyto-

oestrogenic plants and herbs which I have been prescribing include alfalfa, soya beans, red clover, liquorice and beans.

2. I have conducted a number of seminars on phyto-oestrogens and published eight articles on phyto-oestrogens in both professional journals and popular magazines. In 1985, I presented a paper to the National Herbalists' Association of Australia in which I described the use of phyto-oestrogenic plants and herbs as being helpful for treating menopausal symptoms and some categories of premenstrual syndrome. A summary of this seminar is exhibited hereto and marked "NB-1". In 1988, I published an article entitled "Herbal Help to Avoid Menopause Symptoms" in the Journal of *Australian Wellbeing*. A copy of this article is attached hereto and marked "NB-2". In this article I described the use of oestrogen containing plants including red clover and soy beans for the treatment of menopausal symptoms. In 1988, I also published a book, entitled "*The Family Guide to Natural Therapies*" Greenhouse Publications, Richmond. In this book I described the use of phyto-oestrogenic plants in the treatment of menopausal symptoms and some types of premenstrual syndrome. An extract from this book is exhibited hereto and marked "NB-3". In 1995, I published a lengthy article for professional herbalists entitled "Phyto-oestrogens and Compounds that Affect Oestrogen Metabolism - Parts I and II", in the *Australian Journal of Medical Herbalism*. This is a review article which cites some 91 scientific papers, many of which were published before 1992. A copy of this document is exhibited hereto and marked "NB-4".

3. I have read the specification and claims of Australian Patent Application Serial No. 40525/93 (the "Application") and am satisfied that the specification does not disclose any feature or features, either alone or in any combination, which advances the art of natural therapies or isoflavonic phyto-oestrogens and their use in the treatment of human conditions as that art was already developed prior to the priority date of the Application, namely 19 May 1992. It follows that none of the claims of the Application is to subject matter which was new or inventive at that date.

4. Prior to discussing the specification and claims of the Application, I shall consider what was known to me and in my opinion to those of ordinary skill in the art of herbal medicines and pharmaceuticals in Australia before 19 May 1992.

4.1 Phyto-oestrogens are plant hormones that are chemically different from the oestrogens produced in the human body. A particular class of phyto-oestrogens are the isoflavones or isoflavonic phyto-oestrogens. These compounds were first identified and extracted from plants as long ago as the 1920's and 30's. In Australia in the 1940's there was a well publicised and documented outbreak of infertility of sheep grazing on subterranean clover. This observation generated much interest and research in the field of phyto-oestrogens. By way of example I refer to a review article entitled "Potential value of Plants as Sources of New Antifertility Agents II" which was published in the *Journal of Pharmaceutical Sciences* in 1979. A copy of an extract of this document is exhibited hereto and marked "NB-5". This document details some of the then known research on oestrogenic plants and their active principles. I note that this article refers to the isoflavonic phyto-oestrogens genistein, daidzen, biochanin A and formononetin and their identification and isolation from clover. I note that these compounds are the same as those described in the claims of the Application. I also refer to another article by Verdeal and Ryan entitled "Naturally-Occurring Estrogens in Plant Foodstuffs – A Review", *Journal of Food Protection*, Vol 42, No. 71577-583 (1979), a copy of which is attached hereto and marked "NB-6". This is also a review article which describes the existence of oestrogenic substances in plants. The presence of genistein and formononetin in clover is discussed. Reference is also made to the relative affinity of these phyto-

oestrogens for mammalian oestrogen receptors. (See Table 3).

4.2 Prior to 1980 and up to the present time, scientists throughout the world carried out pioneering work on phyto-oestrogens such as measuring and comparing levels of various phyto-oestrogens in the urine of people with different dietary habits. It was observed, in many papers published before 19 May 1992, that the urinary excretion of isoflavonoids correlated with soybean-product intake. The low mortality in breast and prostate cancer in Japanese women and men, respectively, may be due to the high intake of soybean products. This is also a common explanation for the low incidence of hot flushes in Japanese women. In order to illustrate the volume of publications in this field published before 1992, I refer to a Selected Bibliography of Scientific Studies on genistein and other Soya Isoflavones which was published in 1996 in a book entitled "*Soya For Health, The Definitive Medical Guide*". This Bibliography is exhibited hereto and marked "NB-7". Although this bibliography was published in 1996, many of the cited articles were published before 19 May 1992. The results of this research was well known to me and in my opinion also know to those of ordinary skill in the field before 19 May 1992.

4.3 Some selected Examples of the publications which were published before 19 May 1992 are as follows:

(a) An article by H. Adlercreutz entitled "Western diet and Western diseases: some hormonal and biochemical mechanisms and association", *Scandinavian Journal of Clinical and Laboratory Investigation*, 50 Suppl 201, 2023, (1990) a copy of which is exhibited hereto and marked

"NB-8". This document is a review article which describes studies carried out over the previous ten years. At page 15, Adlercreutz concludes "*It is concluded that very similar associations between diet, SHBG, lignans and isflavones, as found for breast cancer seem to exist also with regard to coronary heart disease.*"

(b) A communication by Adlercreutz and Hamalainen the *Lancet*, Vol 339, May 16, 1992. A copy of the document is exhibited hereto and marked "NB-9". This article describes the results of comparing the urinary secretion of isoflavonic phyto-oestrogens in Japanese, American and Finnish women. The excretion of isoflavonic phyto-oestrogens including genistein, daidzen and equol was found to be associated with the intake of soy products such as tofu, soybeans and miso. It was suggested that isoflavanoids in high amounts could have biological effects, especially in menopausal women with low oestrogen levels.

(c) An article by Messina and Barnes entitled "The Role of Soy Products in Reducing Risk of Cancer" in the *Journal of the National Cancer Institute*, Vol 83, April 17, 1991., a copy of which is exhibited hereto and marked "NB-10". This document is a report of a workshop in 1990 in the USA in relation to the effect of soy on cancer. This document cites many earlier publications which describe, amongst other things, the treatment of postmenopausal women by increasing their soy intake to the equivalent of 200mg/day of isoflavones (see page 542, 1st column, last paragraph). This document also cites an animal model designed to study the role of phyto-oestrogens in the

reduction of breast cancer risk. It was observed that soy products including an aqueous alcohol extract of soy flour rich in isoflavones inhibited mammary tumorigenesis (see paragraphs 1 to 4 of page 542).

(d) An article entitled "Oestrogenic effects of plant foods in postmenopausal women", Wilcox et al, *British Medical Journal*, Vol 301, 905-6 (1990). A copy of this document is exhibited hereto and marked "NB-11". This communication describes the results of a study conducted in Melbourne, Australia on the effect of soya flour, red clover or linseed or postmenopausal women. The subject matter of the above document, ie. that the presence of phyto-oestrogens in the diet could be beneficial to human health was well known to me and in my opinion well known to those of ordinary skill in the art in Australia before 19 May 1992.

4.4 Red clover has been used as a herbal remedy since at least the 19th century and its traditional uses include cancer, skin and nerve problems. Red clover as a herb or tea has been prescribed by myself and others skilled in the art in Australia well before 19 May 1992. It was also well known to myself and others skilled in the art in Australia before 19 May 1992 that red clover was a source of isoflavonic phyto-oestrogens.

4.5 It was well known to me and in my opinion also well known to others skilled in the art in Australia that natural products, and in particular those used in herbal medicine, may be extracted from their plant sources by using alcohol and water in varying concentrations. The techniques for making such extracts into liquid, powder or tablet remedies have been known and applied

for decades. To my knowledge, plant extracts were used as far back as 1898. In 1922, an authoritative text listed 32 ways of preparing plant remedies. A copy of an extract from Culbreth, *A Manual of Materia Medica and Pharmacology*, Ecletic Medical Publications, Oregon (1922) is exhibited hereto and marked "NB-12". I refer to paragraphs 6, 9 and 26 which refer to alcoholic solutions and extracts. Single, isolated compounds from plants have been the source of pharmaceutical drugs for many years and they still play a major role in the pharmaceutical industry. For at least 20 years, guaranteed potency natural remedies or phyto-pharmaceuticals have been available that is, natural plant remedies that contain a specified quantity of a specific component or components. An example of such a guaranteed potency natural remedy is an Australian brand of *Ginkgo biloba* tablets which has been labelled as containing 9.6% flavone glycosides.

4.6 It was well known to me and in my opinion well known to others skilled in the art in Australia before 19 May 1992 that isolated plant components give a more pronounced therapeutic effect for specific purposes compared to the whole plant.

5. I now refer to the specification of the Application. Pages 1 to 8 of the specification discuss the state of the art on or before 19 May 1992. In my opinion this description is an accurate representation as to the state of the art in Australia at that time. The Application acknowledges that the isoflavonic phyto-oestrogens or isoflavones, genistein, daidzein, biochanin A and formononetin are known compounds which may be found in a variety of plants including soy and clover. Page 3 of the specification summarizes the known biological effects of phyto-oestrogens. Reference is also made at page 4 to the fact that moderate levels of phyto-oestrogens may "have a beneficial effect on human health". Reference is also

made to the difference in phyto-oestrogen intake in a Japanese and Western diet. The Applicant acknowledges a number of times throughout the specification that increasing the phyto-oestrogen intake in the diet may influence human health. See for example at page 6 lines 20-21 *"Of those dietary components with a potential to influence the aetiology of oestrogen related disease, there is a growing awareness that phyto-oestrogens may have important potential"* and at line 22 *"the beneficial effects of phyto-oestrogens on human health are though to derive from at least two principle functions..."*. At page 16 lines 17 –18: *"Isoflavones are potent anti-oestrogens that could be expected to help or prevent or to successfully treat breast cancer"*. Still further at page 16 lines 25-26: *"Phyto-oestrogens are thought to protect from development of breast cancer"*; at page 17 lines 6-7: *"There is evidence that foodstuffs high in phyto-oestrogens are a suitable alternative to synthetic hormones in this respect, producing alleviation in adverse clinical symptoms"*. At page 7 second paragraph the Applicant states *"In summary, it* ***could be reasonably deduced*** (emphasis added) *that the inclusion of greater levels of foodstuffs high in phyto-oestrogens in the standard diets of men and women in developed countries could be expected to redress a general imbalance of endogenous reproductive hormone metabolism, thereby reducing the predisposition of these community's to the above diseases"*.

6. I now refer to the specification at page 9, first paragraph which states that the present invention concerns obtaining an extract of clover or soy. It would appear to me that the Applicant is claiming to have discovered obtaining active agents from plants in a more concentrated form. However, the isolation of active products from plants was well known to me and in my opinion well known to those of ordinary skill in the art in Australia before 19 May 1992. I therefore cannot see as to where any invention may lie. I note the specification refers to a strategy of making phyto-oestrogens available in a purified form instead of requiring patients to dramatically change their diets to include large amounts of soy products. The herb red clover, in the dried form, may be described as a more concentrated form of phyto-oestrogens than found in foodstuffs such as tofu. As I have mentioned above, red clover has been prescribed by myself and others skilled in the art before 19 May

1992 in order to increase the phyto-oestrogen intake of patients. In summary, I can only reiterate that I cannot see anything new or inventive in providing known active agents in a more concentrated form.

7. I now refer to page 6, third paragraph of the specification which makes reference to the fact that the invention also concerns a method of treatment or prevention of a number of hormone related diseases. Again I cannot see where any invention lies. The Applicant appears to be attempting to claim the discovery of the use of phyto-oestrogens for treating hormone related diseases. However, the use of phyto-oestrogens to treat at least some of these diseases has been practiced by myself and others in the field Australia since before 19 May 1992. Also, I refer to the comments I have made above to the effect that the beneficial effect of a diet high in phyto-oestrogens was well known to myself and in my opinion well known to others skilled in the art before 19 May 1992. Further, I note that the Applicant himself says that it could "*reasonably be deduced*" that increasing phyto-oestrogens in the human diet could reduce the predisposition of communities to "the above diseases". Thus, by the Applicants own admission this aspect of the invention is no more than a "reasonable deduction". Accordingly I cannot see where any invention can lie.

8. I now refer to page 14, paragraph 2 of the specification which states that "dosages" of up to or greater than 1,000 mg may be suitable to treat some conditions" and that "the treatment with the isoflavones should continue for a considerable period, ideally for at least a month and ideally continuously for the whole period for which the health improvement advantages should accrue". In my opinion the effects of 1,000 mg dosage is not established and is significantly above the dosages in the Examples.

On page 8 (lines 23-24), the Application states "...a method for the treatment or prevention of cancer of the prostate, cancer of the bowel...and (lines 29-31) it is stated that "Ideally, the extract is administered regularly on a daily basis over a sufficient period such as at least a month. The health conditions which may be prevented or ameliorated include cancer...". On page 8a (lines 6-8) it is further

stated that "The product also is useful in avoiding or ameliorating cancer in persons. The symptoms produced by these conditions and the general well-being **is also improved** (emphasis added) by the use of these extracts". I note that there is no clinical evidence in the Application for this assertion.

In my opinion, to suggest that a month's treatment might treat, ameliorate or prevent cancer is absurd and highlights the Applicant's lack of clinical experience in treating humans and a failure to appreciate the relationship between the epidemiological data and the known biological activity. In my clinical experience, even simple hormonal imbalances, such as menopausal flushing, may require at least three months' treatment, and often longer. Further, to suggest that dosages of up to 1,000 mg daily of isoflavones may be useful is in my opinion speculative and such quantities may well turn out to be harmful.

9. I now refer to page 15, second paragraph of the specification which says that an important feature of the invention is the ability to provide an accurately determined quality and quantity of active isoflavones when compared with the "*almost impossible task of eating huge amounts of practically inedible foods*". Again the Applicant appears to be claiming the discovery of providing active agents in a form which contains a specified quantity of a specific component or components. There is nothing new in providing a known active agent in a more concentrated form where eating large amounts of a source food may be undesirable. In this respect I refer to the well known garlic and vitamin tablets. For example, Australians have been taking Vitamin C in a purified tablet form since well before 19 May 1992. Providing Vitamin C in tablet form allows the consumption of large amounts of Vitamin C which would be difficult to obtain by eating a source food. Further, providing active agents in a fixed quality and quantity is nothing more than the "guaranteed potency" which I have already made reference to. Providing specified amounts of active agents has been used widely in the pharmaceutical and health food industries in Australia for many years prior to 19 May 1992. The advantage of using guaranteed potency products is that these contain a precise level of one or a small group of phytochemicals. Theoretically, foods should confer more general

advantages and guaranteed potency products more specific advantages. However, based on epidemiological evidence and 15 years' clinical experience with many hundreds of patients, I have observed no major therapeutic difference in using plants or products although tablets or powders may be more convenient for some patients. An advantage of using whole foods is that these contain nutrients and other valuable phytochemicals. Although the level of phyto-oestrogens in foods is somewhat variable we know that people can eat moderate amounts without untoward adverse reactions and the evidence to date is that these foods confer health advantages.

10. I now refer to the Examples of the Application. Example 1 describes harvesting and drying red clover. This describes nothing more than the preparation of red clover herb and tea. Such herbs and teas were available to me and to the Australian public before 19 May 1992. The next step in the example is solvent extraction although I note that the specification states that the extraction step *"can be omitted if desired"*. I understand this to mean that solvent extraction is unnecessary. Thus in my view, this Example describes nothing more then the preparation of traditional red clover herbs which has been used around the world for centuries. In relation to the extraction step, this step is nothing more than standard extraction procedures and in this respect I refer to the comments I have made above that solvent extraction of active agents from plants is standard practice and was well known to me and in my opinion others of ordinary skill in the art in Australia before 19 May 1992.

Example 2 describes separating the hypocotyl from soy beans and grinding the hypocotyls to obtain a powder. This powder was administered to patients in Example 4.

I am not certain about the precise part of the soybean that is being referred to. The germ of a seed is the common word for the embryo. The hypocotyl is defined in botanical texts and biology dictionaries as "a part (my emphasis) of the stem of the embryo or young seedling which is below the cotyledonary node".

I presume the Application refers to the total embryo (or germ) rather than a portion of it or the sprout. Irrespective of the precision of the terminology, it was established long before May 1992 that the embryo of a seed is the reproductive tissue and naturally contains a high proportion of specific plant hormones. In the early stages of growth (a sprout or seedling) the phyto-oestrogen content increases dramatically and this, too, was well-established before May 1992. Furthermore, there is nothing inventive in separating and using the germ of seeds. For example, wheat germ has been sold in health food stores for at least twenty years. There is nothing surprising, new or inventive in respect of Example 2.

Example 5 refers to administering the "inventive composition" which may be "soya or clover". Thus it is unclear to me as to whether in Example 5 hypocotyl powder or clover is administered. It appears to me that the Applicant is suggesting that soy hypocotyls and red clover may be used interchangeably. In relation to the hypocotyl powder I note that at page 12, third last line the ratio of genistein to daidzein in soy hypocotyl is given as 95% daidzein and 5% genistein. Therefore I assume that the powder obtained in Example 2 has this ratio. However, at page 10 lines 10 –11 the specification states that *"it is prudent that both the isoflavones be present in the claimed product in approximately equal proportions"*. I find this statement to be inconsistent with the use of the hypocotyl powder in which the ratio of genistein to daidzein is far from equal. I also note that the soy hypocotyl powder is administered to patients for the treatment of premenstrual syndrome and menopause. As I have mentioned above, I have been treating patients in my clinic since well before 19 May 1992 for these same conditions. I typically prescribe sprouts such as soy, alfalfa and red clover, soy milk, soy products and various phyto-oestrogenic foods and herbs. It is relatively easy to get a therapeutic level of phyto-oestrogens in the diet given that 1 cup soy sprouts contain about 200 mg isoflavonic phyto-oestrogens, 1 glass soy milk contains 40 mg and so on. Furthermore, I note that the Application (Table, page 3) states that a typical Japanese diet contains between 50 – 300 mg a day. The only difference is in the fact that the powder may be in a more acceptable form for consumption. However, I

cannot see how merely using a different form of a plant, i.e. the hypocotyl verses the sprout can in any way be described as inventive.

11. I now refer to the claims of the specification. Claim 1 reads as follows:

> "An isoflavone containing extract of clover or soya prepared by water/organic solvent extraction of said clover or soya followed by recovery of concentrated isoflavones therefrom, which said extract comprises any two or more of the concentrated isoflavones: genistein, daidzein, biochanin A, formononetin and/or their glycosides".

This claim simply describes an extract of soy or clover obtained by well known extraction methods. In relation to the extraction step I refer again to page 18, lines 1-2 of the specification which says that the extraction step can be omitted if desired. As I have previously stated I understand this to mean that the extraction step is optional and that clover may just as well be used in the dried herbal form. I also refer to Example 2 in which the soy hypocotyl is simply ground. There is no solvent extraction step. I also refer to Examples 3 and 4 in which red clover extract and soy hypocotyls are administered to patients respectively and their cholesterol levels are measured. I note that cholesterol levels are reduced in comparable amounts for both the red clover extract and soy hypocotyl. I also note that in Example 5 patients are administered with "the inventive composition" which may be either red clover extract or soy hypocotyl. This supports my understanding that the solvent extraction step makes no significant difference to the efficacy of the composition. As solvent extraction is simply an option and apparently has no advantages over administering the raw product I do not understand the reason for including this feature in the claim. I therefore can see no difference between the product defined in this claim and known soy foods such as soy milk, tofu and soya beans. These foods contain genistein and daidzein and to my knowledge were commercially available in Australia well before 19 May 1992. In any case, I reiterate the comments I have made above to the effect that solvent extraction using an aqueous alcohol solvent is standard procedure in extracting natural products. Still

further, isoflavonic phyto-oestrogens have been extracted from plants products since before 19 May 1992. In this respect I make reference to the following publications:

(i) Farmakalidis E and Murphy P., *Journal of Agricultural Food Chemistry*, Vol 33, 385-389 (1985).

A copy of this article is exhibited hereto and marked "NB-13". This document describes extracting defatted soy flakes with various solvents including 80% methanol (see page 386 in the section entitled "Comparison of Extraction Methods". The extract contained genistein, daidzein and their glycosides, genistin and daidzin (see Table 1). In my opinion the extraction procedure in this document is the same as that defined in claim 1.

(ii) The article by Messina entitled "The role of soy products in reducing the risk of cancer" to which I have previously referred. At page 542, reference is made to a concentrate of an aqueous alcohol extract of soy flour and that this product was rich in isoflavones. I cannot see that there is any difference between claim 1 and the soy concentrate described in this document.

I also reiterate the comments I have made above to the effect that the technology of isolating and concentrating plant flavonoids in remedies existed long before 1992. As I have said previously, such remedies are known in the art as guaranteed potency or phtyopharmaceuticals. It is therefore my opinion that extracting clover or soy with a water/organic solvent adds nothing to what was published and already known to those skilled in the art in Australia before 19 May 1992.

In summary it is my opinion that there is nothing in the extract as defined in claim 1 which in any way advances that art as that art was developed before 19 May 1992. Further, it is my opinion that there is nothing in claim 1 which was beyond the contemplation of the average skilled worker in the art in Australia before 19 May 1992.

12. I now refer to claim 2 which reads:

"An extract according to claim 1, which comprises genistein, daidzein, biochanin A, formononetin in a ratio from 1:2 to 2:1".

First, I am not quite sure as to the ratios which are defined. Does the claim mean that the extract may have genistein in combination with any one of daidzein, biochanin A or formononetin in the defined ratios? From reference to page 10, second paragraph it would appear that a ratio of 1:1 genistein to daidzein is intended. However, as I have previously said, such a ratio conflicts with the use of soy hypocotyl in which the ratio is 5:95 genistein to daidzein. I also note that in each of the Examples in which various amounts of "phyto-oestrogens" are administered, the relative amounts of the phyto-oestrogens are not given. Genistein and daidzein are present in soya sprouts in a ratio of 1:1. See for example Table 8 of my article in the Australian Journal of Herbalism to which I have already referred. I cannot see the relevance of extracting materials in ratios varying from 1:1, 1:2 to 2:1 and 5:95.

In summary, it is my opinion there is nothing in claim 2 which adds anything to that which was published or already known to those of ordinary skill in the art in Australia before 19 May 1992.

13. I now refer to claim 3 which reads:

"An extract according to claim 1 which is an extract from red clover".

This claim defines nothing more than that the extract may be obtained from red clover, a known source of phyto-oestrogens. I reiterate the comments I have made above that red clover is a well known herb which has been administered for its oestrogenic properties in Australia since before 19 May 1992. Accordingly I cannot see how the extract defined in claim 3 adds anything over that which was already known or which was beyond the contemplation of those of ordinary skill in the art in Australia before 19 May 1992.

14. I now refer to claim 4 which reads:

"An extract according to claim 1 which is an extract from soya hypocotyls".

Again, this claim defines nothing more than that the extract may be obtained from a known source of phyto-oestrogens. I reiterate the comments I have made above to the effect that I have been prescribing phyto-oestrogens in the form of sprouts and other phyto-oestrogenic foods and herbs since well before 19 May 1992. I do not believe that the part of the soy which is used makes any difference. I note that this is acknowledged in the specification which says that any legumous plant may be used as a source of phyto-oestrogens (see page 10, third paragraph). Thus, in my opinion, there is nothing in this claim which adds anything over that which was known or used or was beyond the contemplation of the average skilled worker in the field before 19 May 1992.

15. I now refer to claim 5 which reads:

"A composition which comprises an extract according to claim 1 and at least one excipient, diluent, carrier and/or food".

This claim defines nothing more than adding an excipient, diluent, carrier or food to the extract of claim 1. The use of excipients, diluents and carriers in medicines, including herbal medicines is such common practice that I do not

believe further discussion on this point is warranted. As to adding the material to a food, administering medicines including herbal medicines together with food is also common practice and well known before 19 May 1992. In my opinion, this claim defines nothing more than a salad of soy or clover sprouts. Accordingly, I cannot see how this claim adds anything to that which was not already well known to the average skilled worker in the field before 19 May 1992.

16. Claim 6 reads as follows:

> "A pharmaceutical composition which comprises an extract according to any one of claims 1 to 4 in association with one or more excipients, carriers and/or diluents".

I reiterate the comments I have made above in relation to claim 5. There is obviously nothing new in adding excipients, carriers or diluents to pharmaceutical compositions. This has been standard practice in the art since well before 19 May 1992. In my opinion, this claim describes nothing more than red clover tea. Red clover tea was used by myself and to my knowledge others skilled in the art in Australia for many years before 19 May 1992. Thus, I do not believe that there is anything in claim 6 which advances the art as that art was developed at 19 May 1992 or was beyond the contemplation of the average skilled worker in the field before 19 May 1992.

17. Claim 7 reads as follows:

> "A food stuff which contains or is supplemented with an extract according to any one of claims 1 to 4".

I cannot see how this claim differs from claim 5 which says that the composition comprises a food. Accordingly I repeat the comments I have made above in relation to claim 5.

18. Claim 8 reads as follows:

"A pharmaceutical preparation containing an extract according to claim 1 in association with a pharmaceutically acceptable carrier".

I cannot see how this claim differs from claim 6 which defines a pharmaceutical composition association with a carrier. Accordingly I repeat the comments I have made above in relation to claim 6.

19. Claim 9 reads as follows:

"A pharmaceutical preparation according to claim 8 in solid dosage form selected from a tablet, coated tablet, capsule or powder".

This claim merely defines that the pharmaceutical preparation is in solid form. Pharmaceutical preparations have been available in such forms since well before 19 May 1992 and I cannot see how this claim adds anything to any of the previous claims. Accordingly, I believe that there is nothing in this claim which in any way advances the art as that art was developed as at 19 May 1992 or was beyond the contemplation of the average skilled worker in the filed before 19 May 1992.

20. Claim 10 reads as follows:

"A pharmaceutical preparation according to claim 8 wherein said isoflavones are present in an amount of 20mg to 200mg".

This claim does nothing more than describe the isoflavone concentration. I note that the amount of isoflavones in 100g of the foodstuffs in the Table at page 15 of the specification falls within this range. Further, as I have mentioned above I have prescribed phyto-oestrogens in the form of sprouts and other phyto-oestrogenic foods and herbs. This corresponds to about 200mg which

falls within the range in claim 10. Still further, I note that the Messina article at page 542 describes administering 200mg/day phyto-oestrogens to menopausal women. Thus, I cannot see that there is anything new in the range of 20 to 200 mg as defined in claim 10 over that which was known or used in Australia before 19 May 1992.

21. Claims 11 to 21 describe methods of treating or preventing diseases conditions including benign breast disease, premenstrual syndrome, symptoms associated with menopause, cancer of the prostate, cancer of the bowl or elevated blood cholesterol which includes administering an "effective amount" of an isoflavone containing extract obtained by water/organic solvent extraction. I refer to the comments I have made above in relation to the fact that red clover as a herb and soy products have been prescribed by myself and others in Australia before 19 May 1992 for the treatment of menopausal problems. I have also prescribed phyto-oestrogenic plants before 19 May 1992 for treatment of endometriosis, fibrocystic breast disease, lowering cholesterol, treating prostate problems and as an adjunct for cancer and osteoporosis. I do not believe that any of claims 11 to 21 adds anything to that which I was prescribing before 19 May 1992. I also refer to the comments I have made above in relation to the vast body of knowledge which was published before 19 May 1992 in relation to the beneficial effect of phyto-oestrogens on human health. In view of this body of knowledge, only a very small example of which I have referred, I cannot see how there is anything in these claims which in any way advances that art as that art was developed in Australia before 19 May 1992.

22. In summary, the Application appears to have claimed the discovery of the use of isoflavonic phyto-oestrogens for treatment of various human conditions. However, the use of isoflavonic plants, herbs and extracts has been practiced in Australia for treating disease conditions since well before 19 May 1992. Further, the effect of isoflavonic-phyto-oestrogens on human health has been published in some hundreds of documents before 19 May 1992. Thus, I believe that there is nothing in the extracts and method as described in the Application which was new or beyond

the contemplation of the average skilled worker in the art in Australia before 19 May 1992.

I make this solemn declaration by virtue of the *Statutory Declarations Act* 1959 as amended, and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

Declared at Eighth)
this 8th day of Sept 1998)
Before me,) N. Beckham
NANCY BECKHAM

Toni Gunther
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Determination of Isoflavones in Soybean Flours, Protein Concentrates, and Isolates

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The individual and total isoflavone content in commercial soybean protein products has been determined by high-performance liquid chromatography. Dehulled, defatted soybean flours contain the following mean isoflavone content (mg/100 g): daidzin, 61.7; glycitein 7-*O*-glucoside, 12.9; genistin, 119.8; daidzein, 22.8; genistein, 28.7. The same isoflavones were found in soybean protein concentrates and soybean protein isolates but in decreased amounts.

Soybeans contain isoflavones (Naim et al., 1974) that have several known activities, including estrogenic (Drane et al., 1980; Kitts et al., 1980), fungitoxic (Wyman and VanEtten, 1978), and antioxidant (Pratt and Birac, 1979) properties. Because of the ever increasing use of soybean protein products in foods and feeds, it is necessary to know the concentration of these biologically active compounds in various commercial products. Only one report in the literature (Naim et al., 1974) gives any quantitative data on the concentration of isoflavones in soybeans. Therefore, this study has been conducted to determine the amount of these compounds in soybean flours, protein concentrates, and isolates.

MATERIALS AND METHODS

Samples. A dehulled, defatted soybean flour was prepared in the laboratory (Eldridge et al., 1971) from Amsoy soybeans that were grown in 1978. In addition, one sample of commercial soybean meal and eight texturized soybean flours were obtained from various manufacturers. Five commercial samples of soybean protein concentrates (products containing a minimum of 70% protein) were obtained from four manufacturers. Three processors each use a different procedure for the preparation of their concentrates (Circle and Smith, 1972). Five soybean protein isolates (products containing a minimum of 90% protein) were procured from one manufacturer.

Trade names and sources of samples are given in Table I. All samples were ground to pass a 60–80-mesh screen.

Preparation of Extracts. Ground defatted soybean flour was extracted with several solvents to determine the most suitable solvent for dissolving the soybean isoflavones. Solvents investigated were 50%, 80%, and absolute ethanol, 50%, 80%, and absolute methanol, ethyl acetate, and acetonitrile. Refluxing with 80% methanol gave the most reproducible results and maximum extraction.

Table I. Identity of Samples Used in the Study

samples	trade name or description	source[a]
flours		
A	hexane, defatted Amsoy variety, 1978 crop	1
B	Nutrisoy 7B	2
C	unflavored TVP	2
D	Textratein	3
E	Centex 200	4
F	Centex 200 SL	4
G	Centex 400	4
H	Centex 400 SL	4
I	Mira Tex	5
J	Promois III, SL	6
concentrates		
K	Response	4
L	Food protein concentrate	7
M	Pro Con 2000	8
N	Promosoy 100	4
O	GL-801	6
isolates		
P	Edi Pro N	8
Q	Edi Pro A	8
R	Supro 610	8
S	Supro 620	8
T	Supro 710	8

[a] 1, Northern Regional Research Center; 2, Archer-Daniels-Midland Co.; 3, Cargill, Inc.; 4, Central Soya Co.; 5, A. E. Staley Manufacturing Co.; 6, Griffith Laboratories, Inc.; 7, Swift and Co.; 8, Ralston Purina Co.

In the analysis of soybean products, *n*-butylparabenzene, which served as an internal standard, was dissolved in 80% aqueous methanol, and an accurate volume was added to the sample. A 1-g sample with 25 mL of 80% aqueous methanol containing the internal standard was heated (boiling) on a steam bath for 4 h, cooled, and filtered through a Type AP prefilter followed by a Type HA, 0.45-μM filter (Millipore Corp., Bedford, MA).

Chromatography. The previously published chromatographic procedure (Eldridge, 1982) was followed, using a linear methanol gradient from 25 to 50% in 20 min followed by an isocratic hold period of at least 30 min. Response factors for the individual isolated glucosides and

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Table II. Effect of Time on Extraction of Isoflavones from Defatted Soybean Meal (Sample A)

		isoflavone content, mg/100 g, as is					
		80% CH_3OH, reflux, 15:1[b]					45:1,[b]
isoflavone	Naim[a]	1 h	2 h	3 h	4 h	5 h	5 h
daidzin	67	44	53	60	65	66	48
glycitein 7-β-glucoside	39	9	11	12	13	13	9
genistin	197	88	118	131	137	144	130
daidzein	tr	7	7	10	11	7	6
glycitein	tr	tr	1	1	tr	tr	tr
genistein	1	2	2	2	2	1	2

[a] Corrected for an assumed 20% oil content. [b] Solvent: sample ratio.

aglycons were determined based on the internal standard. These response factors were used to calculate the isoflavone and isoflavone glucoside composition of various commercial soybean protein products.

RESULTS AND DISCUSSION

Shown in Figure 1 is a typical elution diagram obtained upon chromatographing an 80% methanol extract of soybean flour. This particular elution pattern is for an extract of Centax 400 flour (sample G).

Table II shows results obtained when a single lot (sample A) of hexane-defatted soybean flour was extracted with hot 80% aqueous methanol for various times at different solvent ratios. Also included from Naim et al. (1974), who used GLC, are data that have been corrected to an oil-free basis by assuming 20% oil. As seen in Table II, there is an increase in the extraction of the isoflavone glucosides with time. The largest increase is in genistin, which goes from 88 mg/100 g of meal in 1 h to 144 mg/100 g of meal in 5 h, whereas changing the solvent ratio from 15:1 to 45:1 and extracting for 5 h do not increase the amount of isoflavone glucoside found. The results indicate that the slow extraction of the isoflavone glucosides is not due to limited solubility and that a 4-h extraction appears to be sufficient for extracting the isoflavones from soybean meal.

Table III gives the amounts of isoflavone glucosides and aglycons measured in several commercial defatted soybean flour products. The glucosides daidzin and genistin account for well over 50% of the total isoflavone found in soybean flours. In sample H, these two isoflavone glucosides account for 75% of the total isoflavones measured.

Table IV gives the results of analyzing five different soybean protein concentrates (products which contain a minimum of 70% protein). Concentrates L and O were prepared by aqueous leaching of defatted soybean flours (Circle and Smith, 1972), and the amount of isoflavone measured in the sample approximates the amount of isoflavone measured in soybean flours (Table III). Concentrates K, M, and N, on the other hand, are prepared by

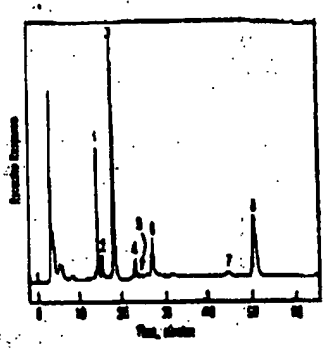

Figure 1. High-performance liquid chromatographic elution diagram of an 80% aqueous methanolic extract of texturized soybean flour. Peaks are (1) daidzin, (2) glycitein 7-β-glucoside, (3) genistin, (4) daidzein, (5) glycitein, (6) genistein, (7) coumesterol, and (8) *n*-butyrophenone.

extracting hexane-defatted soybean meals with aqueous alcohols. This alcohol treatment should remove some of the isoflavones from the meal. The results shown for samples K, M, and N in Table IV indeed show a decrease in the isoflavones.

Table V shows the results obtained when five different soybean protein isolates (products containing at least 90% protein) were analyzed for their isoflavone contents. The majority of the isoflavone measured was genistin, as was observed when soybean flours were analyzed. Although the five soybean protein isolates analyzed are manufactured by different procedures so that the end products have different characteristics, their isoflavone content is fairly constant. About 50% of the total isoflavones in hexane-defatted soybean meal is lost when soybean protein isolate is prepared. The data in Tables III and V show that isoflavone glycosides are preferentially lost in the protein isolation procedure. This decrease in the isoflavone glycosides during protein isolation may be because the glycosides are more soluble than the aglycons in water, which is used for the extraction of soybean protein.

CONCLUSIONS

A high-performance liquid chromatographic procedure has been used to quantitatively measure the isoflavone contents of commercial soybean protein products. The isoflavones in soybeans exist in several forms, i.e., as glucosides, acetyl glucosides, and aglycons. The majority of the isoflavones are present as the glycosides.

In 1980 Drane et al. (1980) showed that rations containing soybean meal prepared for rats caused the uteri of mice to increase in size and concluded soybean meal does have estrogenic activity. Earlier Bickoff et al. (1952) showed that the quantity of genistein and daidzein needed to produce a 25-mg uteri in mice was 5000 and 10 000 μg, respectively. More recent research by Kitts et al. (1980)

Table III. Isoflavone Analysis of Ten Defatted Soy Flours

	mg/100 g, as is, of flour[a]									
isoflavone	A	B	C	D	E	F	G	H	I	J
daidzin	62	61	77	61	49	55	48	77	65	62
glycitein 7-β-glucoside	16	13	22	13	12	13	11	15	6	7
genistin	127	123	146	119	102	58	98	154	142	129
daidzein	48	8	37	46	38	31	17	33	46	27
glycitein	tr	2	3	tr	3	2	tr	tr	tr	tr
genistein	40	4	21	46	27	19	21	26	38	25
total	295	210	306	285	229	178	196	305	296	250

[a] Average of two replicates. Relative standard error per mean is 9.6%. Least significant ratio (0.05 level) of two means is 1.2.

Table IV. Isoflavone Analysis of Five Soybean Protein Concentrates

isoflavone	mg/100 g, as is, of concentrate[a]				
	K	L	M	N	O
daidzin	3	59	9	4	76
glycitein 7-β-glucoside	1	22	2	1	12
genistin	4	134	19	6	191
daidzein	11	20	12	2	11
glycitein	1	tr	tr	1	4
genistein	1	22	1	2	22
total	21	247	43	16	317

[a] Average of two replicates. Relative standard error per mean is 13.7%. Least significant ratio (0.05 level) of two means is 1.6.

Table V. Isoflavone Analysis of Five Soybean Protein Isolates

isoflavone	mg/100 g, as is, of isolate[a]				
	P	Q	R	S	T
daidzin	16	14	23	20	20
glycitein 7-β-glucoside	8	4	4	8	6
genistin	67	59	80	65	55
daidzein	6	12	18	10	21
glycitein	2	1	2	1	3
genistein	22	13	18	6	17
total	116	103	145	105	132

[a] Average of two replicates. Relative standard error per mean is 13.1%. Least significant ratio (0.05 level) of two means is 1.6.

indicates that lesser amounts of genistein may be needed to cause an effect in rat uteri. Our studies show low levels of daidzein and genistein in soybean protein products but large amounts of the isoflavone glucosides. The total isoflavone glucosides and aglycons measured in this study in hexane-defatted soybean meal appears to be approximately 2500 μg/g. Further research is needed to study these soybean constituents as a source of estrogenic response in animals.

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Effect of pH on the Extraction and Fractionation of Dry Matter and Crude Protein from Coastal Bermuda Grass and White Clover

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Freeze-dried Coastal Bermuda grass (CBG) and white clover (WC) were extracted at pHs ranging from 4 to 10 and fractionated into four distinct fractions: chloroplastic (CHL), cytoplasmic (CYT), nonprotein nitrogen (NPN), and residue (RES). Dry matter (DM) and crude protein (CP) distributions in the fractions were influenced by pH. At pH 4, the greatest amount of CHL protein was extracted from CBG while the least amount was extracted from WC. At pHs ranging from 6 to 10, the CHL CP extracted remained constant for each forage with WC having twice the CHL CP as CBG. CYT CP extractability exhibited a quadratic effect ($P < 0.001$) due to pH; the pH optima for extraction of CYT proteins occurred at pHs 7 and 8 for CBG and WC, respectively. The amounts of CYT CP extracted from CBG and WC at their optimal pH were equivalent. The NPN fractions increased in CP with increasing pH while the CP in the RES fractions decreased with increasing pH for both forages. In general, the DM distribution paralleled the CP distribution.

The economical production of leaf protein concentrates from forages is desirable since forages can yield more dry matter and crude protein than any other crop (Pirie, 1978). The fractionation of forage proteins into green chloroplastic fractions for use in animal feeds and nearly white cytoplasmic fractions for human use has increased the importance of forages as sources of protein (Subba Rau et al., 1968; Evans et al., 1974; Horigome, 1977; Hanna and Ogden, 1980). Consequently, many different extraction and fractionation procedures have been described (Spencer et al., 1970, 1971; Pirie, 1971; Fishman and Burdick, 1977; Ostrowski, 1979) in an attempt to efficiently extract proteins from different plants. Chloroplastic and cytoplasmic proteins have been separated mainly on the basis of differential heat treatment of the expressed plant juices (Byers, 1967; Lexmüller et al., 1970; de Fremery et al., 1972; Edwards et al., 1975; Miller et al., 1975). These extraction

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